D-LACTIC ACIDOSIS: STUDIES IN NEONATAL DIARRHEA AND EFFECTS OF PROCESSING AND STORAGE ON LACTATE CONCENTRATIONS IN BLOOD

A Thesis Submitted to the College of

Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of Master of Science

in the Division of Nutrition and Dietetics,

College of Pharmacy and Nutrition

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ABSTRACT

D-lactate is a significant contributor to acidosis in diarrheic calves. To ensure an accurate measure of D- and L- lactate, this study investigated storage dependent changes in D- and L-lactate concentrations in plasma and serum over time with or without prolonged contact with blood cells. Further studies investigated if D-lactic acidosis occurs in children with diarrhea and if a fecal threshold exists in the gastrointestinal tract at which D-lactate enters the systemic circulation in diarrheic calves.

To determine the stability of D- and L-lactate, blood was obtained from eleven healthy calves. D- and L-lactate concentrations in all samples separated following collection (serum, plasma, spiked, untreated) were stable up to 48 hours. L-Lactate concentrations increased significantly ($P < 0.05$) by 74.4%, 39.4%, and 40.2% in untreated and spiked serum and spiked plasma respectively at 48 hours when stored in contact with blood cells. D-Lactate concentrations in untreated serum stored in contact with blood cells increased significantly, by 82.3%, at 48 hours. For accurate measurements of D- and L-lactate, serum or plasma should separated from blood cells as soon as possible but can thereafter be stored at 4°C for up to 48 hours.

To determine whether D-lactic acidosis occurs in children with acute diarrhea, blood samples were obtained from nine children with acute diarrhea, ten months to three years of age. No cases of D-lactic acidosis (> 3 mmol/L) were found, however, D-lactate concentrations ($\bar{x}$ = 0.15) were found to be higher when compared to healthy children.

To determine if a fecal D-lactate threshold exists at which D-lactate enters the blood in diarrheic calves, blood and fecal samples were obtained from 27 calves. The linear regression between fecal D-lactate and serum D-lactate was statistically significant however the range of D-lactate concentrations obtained in this study was not large enough to confirm the fecal threshold at levels previously reported.

Collectively, these studies contribute to the knowledge of D-lactate, and D-lactic acidosis, by determining the stability of D- and L-lactate for accurate measurement, revealing elevated D-lactate concentrations in diarrheic children in comparison to healthy children, and further investigation of a potential fecal D-lactate threshold.

ACKNOWLEDGMENTS

I would foremost, like to thank my supervisor, Dr. Gordon Zello, for his guidance and support throughout my graduate program. The knowledge and skills you provided me with through this work is invaluable. I would also like to thank the members of my Advisory Committee: Drs. Jane Alcorn, Garth Bruce, and Susan Whiting for providing expertise on all aspects of this work. Thanks to the physicians and nurses at Royal University Hospital for their collaboration, patient recruitment, and sample collection and to Dr. Katharina Lohmann, Kim Burker, and the staff at the Dairy Barn for their work with the calves. This work would not have been possible without research funding from NSERC and Vitamin Settlement Grant Canada and personal support from the University of Saskatchewan, Elizabeth Helen McLeod Scholarship Fund, and Global Partners II/IDRC Student Scholarship.

I would also like to thank my lab-mates: Theodosia Phillips, Marc Morris, Abdulla Alemmari and Lindsay Tumback for their daily support and thoughtful feedback. I express sincere gratitude to my friend, and colleague, Binbing Ling, who patiently worked with me to develop the skills required to complete this study.

Lastly, I would like to thank my friends and family. In particular, I would like to thank Kelly Delorme-Greyeyes who supported me in this journey from day one. Your patience, love, and understanding are greatly appreciated. Thanks to my mother Vivian Wright, my sister Audra Wright-Hoffman, Liam, and Tate for their support and for teaching me the importance of fun, and the power of laughter, especially during those long work days.

This thesis is dedicated to my father, William Walter Wright, whose constant love, support, and commitment to life-long learning has been my inspiration. Thank-you Dad.

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LIST OF ABBREVIATIONS, SYMBOLS AND ACRONYMS

%	percent or of each hundred
±	plus or minus
˚C	degrees Celsius
AIDS	acquired immune deficiency syndrome
AG	anion gap
AGA	American Gastroenterological Association
AMP	adenosine monophosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BSFS	Bristol stool form scale
C	contact
CDC	Center for Disease Control
CE	capillary electrophoresis
CoA	coenzyme A
D-LDH	D-lactate dehydrogenase
et al	*et alia*; and others
EAEC	enteroaggregative *Escherichia coli*
EHEC	enterohemmorrhagic *Escherichia coli*
EIEC	enteroinvasive *Escherichia coli*
ETEC	enterotoxigenic *Escherichia coli*
FAO	Food and Agriculture Organization
g	gram(s)
g	gravitational (g)-force
Glo1	glyoxylase I
Glo2	glyoxylase II
GMP	guanosine monophosphate
GS	gas chromatography
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
HU	Hawassa University
i.e.	*id est*; that is
iPAG	disease activity index pediatric gastroenteritis
L-ALT	L-alanine animotransferase
LDH	lactate dehydrogenase
MS	mass spectrophotometer
µM	micromole(s) per litre
MCT	monocarboxylate transporter(s)
mEq/L	milliequivalent(s) per litre

mg	milligram(s)
mL	millilitre(s)
mmol/L	millimole(s) per litre
mM	millimole(s) per litre
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced form)
ORT	oral re-hydration therapy
PDH	pyruvate dehydrogenase
PedsER	pediatric emergency room
p	p-value or probability
pH	negative logarithm of hydrogen ions to the base 10
rpm	revolutions per minute
r	Pearson product-moment correlation coefficient
RBC	Red blood cells
RUH	Royal University Hospital
S	Seperated
SBS	short bowel syndrome
SD	standard deviation
UNICEF	United Nations Children's Fund
USDA	United States Department of Agriculture
vs.	versus
WHO	World Health Organization
µl	microliter(s)
µM	micromole(s) per litre

CHAPTER 1
INTRODUCTION

1.1 Rationale

Despite much global effort, advances in research, and updated clinical management guidelines, diarrhea continues to be a leading cause of mortality and morbidity in animals and humans worldwide. In neonatal calves, diarrhea accounts for more than 50% of unweaned heifer deaths (United States Department of Agriculture [USDA], 2008) and results in significant economic losses in the beef and dairy industries (Barrington, Gay & Evermann, 2002; Gunn, Naylor, & House, 2009). In children, diarrhea is the second leading cause of death due to infectious disease and kills more children than acquired immune deficiency syndrome (AIDS), malaria, and measles combined (United Nations Children's Fund [UNICEF] & World Health Organization [WHO], 2009).

Diarrhea is a complex, multi-factorial illness and, in both humans and neonatal calves, the majority of cases are due to infections of the gastrointestinal tract by bacteria, viruses, and/or parasites (Guandalini, 2005; Holland, 1990). Regardless of the species, cause, and pathophysiology, diarrhea can lead to serious clinical consequences including electrolyte disturbances, dehydration, and metabolic acidosis (Guandalini, 2005; Gunn et al., 2009). Metabolic acidosis is defined as a disturbance to the body's acid-base balance, which results in a decrease of blood pH < 7.35 (Ayers & Warrington, 2008). Metabolic acidosis can lead to serious clinical complications and, if left untreated, can lead to death. Initially, acidosis commonly observed in animals and humans with diarrhea was thought to be due to a loss of bicarbonate in the feces and overproduction of L-lactate in the tissues as a result of dehydration (Kasari, 1999; Perez, Oster & Rogers, 1987). Research has now shown that acidosis can occur in the absence of dehydration and that D-lactate, a physiological isomer of L-lactate, is a significant contributor to acidosis in diarrheic calves (Kasari & Naylor, 1984; Omole, Nappert, Naylor & Zello, 2001; Ewaschuk, Naylor, Palmer, Whiting & Zello, 2004). D-lactate is produced primarily by microorganisms in the gastrointestinal tract with very small amounts produced endogenously via the methylglyoxal pathway in the liver (Uribarri, Oh, & Carroll, 1998).

Clinical symptoms associated with D-lactic acidosis are similar in both humans and animals and include neurological disturbances such as abnormal motor coordination, including weakness

and ataxia (Kasari & Naylor, 1984; Kasari & Naylor, 1986; Lorenz, 2004; Lorenz, Gentile & Klee, 2005; Lorenz 2009; Abeysekara, Naylor, Wassef, Isak, & Zello, 2007; Coronado, Opal, & Yoburn, 1995; Uribarri et al., 1998). Slurred speech, blurred vision, confusion, dizziness, lack of concentration, delirium, and abusive/hostile behaviours has also been documented in humans (Uribarri et al., 1998; Hove & Mortensen, 1995; Stolberg et al., 1982; Al Chekakie, Al Kotoub, & Nielsen, 2004). In severe cases, D-lactic acidosis can result in acute encephalopathy and coma (Grünert et al., 2010; Al Chekakie et al., 2004; Htyte, White, Sandhu, Jones, & Meisels, 2011). Symptoms vary in duration and appear to be non-specific; therefore, early detection and diagnosis of elevated blood lactate levels is important for initiating appropriate treatment including fluid and electrolyte replacement to correct the acidosis, temporary restriction of carbohydrates, and use of antibiotics to minimize D-lactate producing bacteria and restore gastrointestinal bacterial flora (Uchida et al., 2004; Al Chekakie et al., 2004).

Pre-analytical conditions including blood storage and processing may influence the stability of lactate. Studies have shown that delays in processing blood can result in significant overestimations of lactate levels due to ongoing glycolysis (Vaught, 2006; Calatayud & Tenías, 2003; Boyanton & Blick, 2002). Most research on the stability of lactate focuses on total and/or L-lactate concentrations; however, little is known about the stability of D-lactate. As high levels of D-lactate are associated with physical and mental changes in diarrheic calves, determination of the effects of pre-analytical storage and processing on blood D- and L-lactate is needed to ensure a reliable measurement of D-lactate concentrations.

In humans, D-lactic acidosis (serum concentration > 3mmol/L) is most often reported as a rare clinical complication of short bowel syndrome (SBS). Carbohydrate malabsorption, a complication of SBS, may result in excessive bacterial gastrointestinal fermentation, which creates an environment that favours the growth of anaerobic bacteria that produce D-lactate. When D-lactate accumulates in the gastrointestinal tract, it is absorbed into the blood and may result in D-lactic acidosis (Hove & Mortensen, 1995; Ewaschuk, Naylor, & Zello, 2005; Petersen, 2005). Identifying D-lactic acidosis in humans with acute diarrhea, in absence of SBS, has been poorly examined. This warrants investigation because, similarly to SBS, carbohydrate malabsorption in human infants has been associated with diarrhea and metabolic acidosis with little detectable bicarbonate in the feces (Sack, Rhoads, A. Molla, A.M. Molla, & Wahed, 1982).

It is thus expected that elevated blood D-lactate concentrations, and D-lactic acidosis, may occur in children with diarrhea.

Elevated fecal D-lactate concentrations observed in diarrheic animals provides evidence that it is the production of D-lactate via gastrointestinal bacterial fermentation that contributes to D-lactic acidosis (Omole et al., 2001; Ewaschuk et al., 2004a). Recently, research in diarrheic lambs and calves suggests that elevated levels of D-lactate begin to appear in the blood only after high levels are produced in the gastrointestinal tract. Thus, a potential gastrointestinal threshold may exist for the absorption of D-lactate into the blood of diarrheic neonatal animals (Abeysekara, 2009; Zello et al., 2009). Further investigation into a potential fecal D-lactate threshold is warranted in which more diarrheic animals with a wide range of diarrhea severity are included. Identifying elevated fecal D-lactate levels, early on, in clinical cases of diarrhea may prove to be a useful biomarker to predict, and ideally prevent, D-lactic acidosis.

1.2 Hypothesis

This project tested the following three hypotheses:

- As cellular metabolism continues following blood sample collection, blood D- and L-lactate concentrations increase over time and in response to different processing procedures
- Children (≤ 5 years of age) with acute diarrhea and metabolic acidosis have elevated levels of blood and fecal D-lactate concentrations
- Supra-physiological blood D-lactate concentrations appear after sufficiently high levels of D-lactate are produced by microbial action in the gastrointestinal tract in neonatal calves (≤15 days of age)

1.3 Objectives

The objectives of this study were:

- To examine the stability of D- and L-lactate concentrations in calf blood samples:
    a) After prolonged contact of serum and plasma with blood cells
    b) After immediate separation of serum and plasma from blood cells via centrifugation
- To determine if D-lactic acidosis occurs in children (≤ 5 years of age) who come to hospital with acute diarrhea and metabolic acidosis
    - To investigate the blood parameters that define metabolic acidosis and determine

concentrations of D-lactate, L-lactate, and pyruvate in children with acute diarrhea

- To examine a wide range of diarrhea severity to determine if a fecal threshold exists in the gastrointestinal tract at which D-lactate enters the systemic circulation in neonatal calves with diarrhea

CHAPTER TWO
LITERATURE REVIEW

2.1 Diarrhea Pathophysiology

The gastrointestinal tract has a great capacity to handle large volumes of fluid. In humans, approximately seven to ten litres of fluid (oral fluids, saliva, small intestine secretions and gastric, bile and pancreatic juices) enters the gastrointestinal tract every 24 hours with very little loss, approximately 1%, in the feces (Johnson, 2007). The gastrointestinal tract maintains homeostasis by regulating the absorption and secretion of both fluids and electrolytes (Johnson, 2007). This regulation of fluid is critical to ensure normal gastrointestinal function including nutrient digestion and absorption (Barrett, 20006; Acra & Ghishan, 1996). When there is a disturbance to the balance between intestinal absorption and secretion processes, diarrhea occurs. Diarrhea can result from various mechanisms and/or a combination of mechanisms including increased intestinal secretion of fluid and electrolytes; decreased absorption of fluid, electrolytes and nutrients; and/or motility disturbances (Thapar & Sanderson, 2004; Colbère-Garapin et al., 2007; Guandalini, 2005; Acra & Ghishan, 1996; Casburn-Jones & Farthing, 2004).

Clinically, diarrhea can be classified as acute or chronic. Acute diarrhea has a sudden onset, generally resolves within fourteen days, and is usually due to an infectious agent. Chronic diarrhea has been defined as loose stools, with or without an increase in frequency, which lasts more than four weeks. Many causes of chronic diarrhea exist and include inflammatory bowel disease, malabsorptive syndrome, and chronic infections (American Gastroenterological Association [AGA], 1999; Fine & Schiller, 1999). In addition to the duration of the diarrheal episode, fecal characteristics help guide the treatment and management of diarrhea and is classified as non-inflammatory or inflammatory (Baldi, Bianco, Nardone, Pilotto, & Zamparo, 2009).

2.1.1 Non-Inflammatory Diarrhea

Non-inflammatory diarrhea is characterized by large volume, watery, and liquid stools. It is caused by decreased gastrointestinal absorption of water and electrolytes and/or increased secretion of water and electrolytes into the gastrointestinal tract (Thapar & Sanderson, 2004; Navaneethan & Giannella, 2008). A loss in the ability of the lumen to absorb water and

electrolytes may result from the loss of part of the intestinal absorptive area (i.e. short bowel), decreased intraluminal digestion (i.e. pancreatic insufficiency), decreased enterocyte cellular absorptive function (i.e. abnormalities in intestinal transporters) and decreased intestinal transit (i.e. drugs, toxins, stress) (Thapar & Sanderson, 2004). Increased secretion can be caused by an increase in the production of secretory cells as in such conditions where enterocytes and/or absorptive intestinal villi are damaged (i.e. food allergies, celiac disease, viruses). Increased secretion may also be a result of an up-regulation of secretory pathways activated by secretory enterotoxins (Farthing, 2000; Thapar & Sanderson, 2004).

Enteric pathogens in non-inflammatory diarrhea usually target the small intestine. Such pathogens include rotavirus; norovirus; enterotoxigenic *Escherichia coli* [ETEC]; *Vibrio cholera; Staphylococcus aures*; *Clostridum perfringens*; *Giardia Lamblia*; and *Cryptosporidium parvum.* These pathogens tend not to invade the intestinal wall but attach to the mucosa and interfere with normal enterocyte absorption and secretion. Several of these pathogens will also secrete enterotoxins that stimulate increased intestinal secretions (Colbère-Garapin et al., 2007; Navaneethan & Giannella, 2008).

Non-inflammatory diarrhea can be further classified as osmotic or secretory (Sellin, 2001). Osmotic diarrhea occurs when there is a decrease in the absorption of osmotically active solutes in the gastrointestinal tract and results in water being drawn into the lumen at a rate proportional to its concentration. This excess water may exceed the re-absorptive capacity of the colon and diarrhea occurs (Castro-Rodrígues, Salazar-Lindo, & León-Barúa, 1997; Thapar & Sanderson, 2004; Acra & Ghishan, 1996; Sellin, 2001). In contrast, in secretory diarrhea the net secretion of water into the intestinal lumen is caused by the intestinal epithelium itself as a result of an imbalance between electrolyte secretion and absorption (Baldi et al, 2009; Guarino, Buccigrossi & Armellino, 2009; Sellin, 2001). Changes may be mediated by cyclic adenosine monophosphate (AMP) or cyclic guanosine monophosphate (GMP), calmodulin, and changes in protein kinase activity. Intestinal cells are not damaged. Rather, cellular membrane pumps are altered and secretion of chloride, sodium, and potassium increases (Gunn et al., 2009).

2.1.2 Inflammatory Diarrhea

Inflammatory diarrhea can be characterized by small volume, mucoid, bloody feces and is caused by enteric pathogens that target the colon or distal ileum (Navaneethan & Giannella,

2008). Enteric pathogens associated with inflammatory diarrhea secrete cytotoxins (enteroaggregative *Escherichia coli* [EAEC], enterohemmorrhagic *Escherichia coli* [EHEC] and *Clostridium difficile*), and/or invade the intestinal wall (*Shigella spp.*, *Campylobacter* spp., *Salmonella* spp., *Yersinia* spp., enteroinvasive *Escherichia coli* [EIEC] and E. *histolytica*), and cause an acute inflammatory response in the mucosa (Colbère-Garapin et al., 2007; Navaneethan & Giannella, 2008). Diarrhea is thought to be a result of increased intestinal secretion stimulated by cytotoxins and inflammatory mediators and/or mucosal damage. Histologically, due to inflammation, ulceration of the mucosa may be observed and, clinically, stools may test positive for fecal leukocytes (Navaneethan & Giannella, 2008).

2.2 Diarrhea in Humans and Neonatal Calves

2.2.1 Pediatric Diarrhea

2.2.1.1 Global Impact of Diarrhea

Diarrheal diseases continue to be a global public health concern, especially in children, as these diseases contribute significantly to human morbidity and mortality. Although the mortality rate of children under five years of age has fallen within the last twenty years due to oral re-hydration therapy (ORT), diarrhea continues to be a leading cause of child death (UNICEF & WHO, 2004). According to Black et al. (2010), diarrhea accounts for an estimated 1.336 million deaths globally, approximately 15% of total child deaths, with 51% of these deaths occurring in India, Nigeria, Afghanistan, Pakistan, and Ethiopia. Worldwide, diarrhea is the second cause of death due to infectious agents following pneumonia and kills more children than AIDS, malaria, and measles combined (UNICEF & WHO, 2009). Kosek, Bern, & Guerrant (2003) estimated a global median incidence of 3.2 episodes of diarrhea per child-year in developing countries. Reduction of diarrhea-related mortality burden is crucial towards achieving the United Nation's Millennium Development Goal #4 to, "reduce by two thirds, between 1990 and 2015, the mortality rate of children under five" (United Nations, 2002).

In both developing and developed countries, diarrheal diseases exerts pressure on existing health care services, finances, and ultimately affects the child's quality of life. Children living in developing countries are more likely to have a higher number of diarrheal episodes, severe episodes with dehydration, and higher death rate compared to those children living in a middle or high-income country (O'Ryan, Prado, & Pickering, 2005). The range in the number of diarrheic

episodes is dependent on a variety of risk factors including poor infrastructure, crowding, exposure to farm animals, lower standards in food handling and hygiene, decreased accessibility to health care, and lower educational levels (O'Ryan et al., 2005). Although, morbidity and mortality rates associated with diarrhea may be less in developed countries, diarrheal diseases are still very much a concern. In the United States, between the years of 1993-2003, 1 in 6 children were hospitalized due to a diarrheal-illness (Fischer, et al., 2007). According to Malek et al. (2006), diarrhea is associated with 150 000 to 170 000 hospitalizations in the United States annually and accounts for approximately 13% of all hospitalizations among children with a median charge of $2051 US dollars per hospitalization (Malek et al., 2006).

2.2.1.2 Defining Pediatric Diarrhea

In children, diarrhea has been formally defined as an increase in stool fluidity above a normal value of approximately 10 mL/kg per day (Guandalini, 2005; Thapar & Sanderson, 2004). A more practical assessment and diagnosis used in the clinical setting is an unusual increase of liquid and watery stool at least three times within a 24 hour period (Guandalini, 2005; O'Ryan et al., 2005) with a particular emphasis on the observation of a change in stool consistency (WHO, 2005; O'Ryan et al., 2005). A diarrheal "episode" begins with the first 24 hour period that meets the definition of diarrhea and ends with the last diarrhea day, which is defined as at least two days that do not meet the definition of diarrhea (Lima & Guerrant, 1992).

2.2.1.3 Etiology

Many causes of pediatric diarrhea exist with the majority of cases due to infections of the gastrointestinal tract. Causes of diarrhea vary with the location, time of year, and the population group studied (Guandalini, 2005; Dennehy, 2005). An increasing variety of enteric pathogens are associated with diarrheal diseases, which can be classified into three microbial groups: bacteria, parasites, and viruses (Guandalini, 2005; Dennehy, 2005). In the United States, 35% of diarrheal-associated hospitalizations among children under five years of age are attributed to viral agents, 5% are bacterial, <1% parasites, and 62% are of an unspecified etiology (Malek et al., 2006). In addition to a large number of viruses, developing countries and areas that have poor sanitation and food and water contamination have a high risk of infection caused by enteric bacteria and/or parasites (O'Ryan et al., 2005). More specifically, *Shigella* spp. contributes to

15% of diarrheal associated mortality (Thapar & Sanderson, 2004). Other bacteria known to cause diarrhea in developing countries include pathogenic *Escherichia Coli*, *Campylobacter*, *Yersinia,* and *Salmonella* species. *Vibrio cholerae* is a concern where sanitation is poor (Thapar & Sanderson, 2004).

Rotavirus is one the most common pathogens causing diarrhea in children under five years of age in both the developed and developing world (Fischer et al., 2007). Worldwide, it causes approximately 600,000 to 875,000 deaths annually and is responsible for 6% of deaths (Dennehy, 2005). Rotavirus is widespread and most severe in developing countries in which it has been estimated to be responsible for 60% of diarrheal diseases (Thapar & Sanderson, 2004). Death associated with this virus often results from dehydration. Similar to other pathogens that cause infectious diarrhea, rotavirus infects the villous epithelium of the small intestinal mucosa (villous atrophy), which leads to nutrient malabsorption, increased fermentation in the colon, and results in watery diarrhea (Cohen, 1991). Vaccines are available but remain costly (Colbère-Garapin et al., 2007).

The pathogens responsible for enteric infections are commonly ingested through contaminated food, water, or via person-to-person contact (Carroll & Reimer, 2000). The risk of developing an infection with several enteric pathogens increases where there is a lack of potable water (O'Ryan et al., 2005). The likelihood of developing an infection as a result of being exposed to the pathogens mentioned above is dependent on several factors: frequency of exposure; the number of pathogens ingested; and host defence factors including gastric acidity, intestinal motility, normal gastrointestinal flora, and host's intestinal immunity (Carroll & Reimer, 2000; Cohen, 1991).

2.2.1.4 Prevention and Treatment

In 2009, UNICEF and WHO published a report detailing a 7-point strategy for diarrhea. This report includes a treatment package, which aims to reduce death due to diarrhea, and prevention package, which aims to reduce the total number of cases of diarrhea (UNICEF & WHO, 2009). Prevention strategies are vital in managing diarrheal disease. Strategies for prevention are developed to reduce the child's exposure to those pathogens that cause diarrhea directly and to reduce the child's susceptibility to diarrhea through improved health and nutrition. More specifically, UNICEF & WHO (2009) focus on five prevention elements

including rotavirus and measles vaccination; breastfeeding and vitamin A supplementation; promotion of hand washing with soap; improved water quality and quantity; and community-wide sanitation promotion.

Treating diarrhea is based on clinical symptoms including the type of diarrhea (watery vs. bloody), duration (acute vs. chronic) and degree of dehydration. The most common cause of hospitalization of infants and children with acute diarrhea is due to dehydration. Infants and children with acute diarrhea are at a higher risk of having more severe dehydration due to a higher body surface-to-body ratio, higher metabolic rate, smaller water reserves, and their dependency on others for fluid (King, Glass, Bresee, & Duggan, 2003). Ultimately, the main treatment goals are to prevent and/or treat dehydration and electrolyte disturbances, replace ongoing losses, shorten the duration of the illness, and continue normal feeding (Guarino et al., 2008; Cashburn-Jones & Farthing, 2004; Colletti, Brown, Sharieff, Barata, Ishimine, 2008). Fluid and electrolyte replacement can be administered orally (i.e. oral rehydration therapy [ORT]) or intravenously.

Oral rehydration therapy includes rehydration and maintenance fluids with oral rehydration solutions (ORS) combined with continued feeding (Victora, Bryce, Fontaine & Monasch, 2000; King et al., 2003). The scientific basis for ORS came from the discovery that sodium transport was mediated by glucose transport in the small intestine. Transport across the lumen is made possible by sodium glucose co-transporter 1 (SGLT1). Glucose can then be transported into the blood via glucose transporter type 2 (GLUT2) (King et al, 2003). As this coupled transport mechanism does not appear to be impaired in infectious diarrhea the use of glucose-electrolyte solutions are shown to be effective in treating diarrhea-associated dehydration. This discovery was initially investigated in cholera; however, ORS has been shown to be an effective treatment in over 90% of cases despite the cause of diarrhea (Booth, Levine, & Harries, 1984; WHO, 2005). Although various formulations of ORS exist, the WHO recommends the use of a reduced osmolarity ORS solution as it decreases the need of intravenous fluid when compared to higher osmolarity solutions. The ORS recommended by WHO has a total osmolarity of 245 mOsm/L and contains 75 mmol/L sodium; 65 mmol/L chloride; 75 mmol/L glucose; 20 mmol/L potassium; and 10 mmol citrate. This ORS solution provides water and electrolytes to replace loss in acute diarrhea; citrate to prevent or correct base deficits; and glucose to allow for maximum absorption of sodium and water. When compared to

other reduced osmolarity solutions, this formulation reduces stool output by 20% and number of vomiting episodes by 30% (WHO, 2005).

A review by Hartling et al. (2006) concluded that there are no clinically important differences between ORT and intravenous treatment in gastroenteritis although ORT did have a higher risk of paralytic ileus and intravenous fluids increased the risk of intravenous therapy (i.e. phlebitis). Children with mild to moderate dehydration should be treated with ORT with ORS solution first, and if treatment should fail, then intravenous fluids should be used. Although WHO, American Academy of Pediatrics (AAP) and the Center for Disease Control (CDC) strongly recommend the use of ORT in mild to moderate dehydration it continues to be underused, especially in North America (Bender, Ozuah, & Crain, 2007). Conversely, in developing countries, ORTs are the main form of treatment due lack of access and costs associated with intravenous treatments (Colletti et al., 2008). In addition to ORT, UNICEF & WHO (2004) recommends daily 20 mg zinc supplements for ten to fourteen days in children with acute diarrhoea, and 10 mg per day for infants under six months old, to reduce the duration and severity of diarrhea and to prevent reoccurring episodes. Other pharmacological agents are available; however, some agents including anti-motility drugs (e.g. loperamide), are not recommended in infants and children as serious side effects including shock, ileus, nausea, lethargy and death have been reported (King et al., 2003; Li, Grossman, & Cummings, 2007).

Much attention has been given to the potential role of probiotics in treating and managing pediatric diarrhea although the mechanisms by which they work are not as fully understood. The gastrointestinal tract contains over 400 species of bacteria, mainly anaerobic, which play an important role in the functioning of the mucosal barrier and contribute to overall health by producing nutrients, protecting the gastrointestinal tract against enteric pathogens, and play a role in the body's immune system (Isolauri, et al., 2002; Thomas & Greer, 2010; Balamurugan et al., 2008). In acute diarrhea, enteric pathogens may disrupt the gastrointestinal tract environment and, as a result, change the normal microbial flora. Balamurugan et al. (2008) observed fecal microbial changes in diarrhea including significantly lower numbers of *Bacteroides-Prevotella*, a predominant fecal anaerobic bacteria, and *Eubacterium rectal* and *Faecalibacterium prauznitizii*. Changes in the gastrointestinal flora may lead to a decrease in the predominant bacteroides species and support the growth of acid producing bacteria such as lactic acid (i.e. *Lactobacillus)*. This may result in an increased production of both D- and L-lactate (Al Chekakie et al., 2004;

Uribarri, et al., 1998). The physiological mechanism by which the gastrointestinal tract produces unusually high amounts of D-lactate is further described in Section 2.4.2.3.1.

Probiotics have been defined by the Food and Agriculture Organization (FAO) & WHO (2001), as a supplement or part of food, as "live microorganisms which when administered in adequate amounts confer a health benefit on the host." A systematic review by Allen, Martinez, Gregorio, and Dans (2010) reports the use of probiotics in acute diarrhea due to enteric pathogens may help to restore normal gut flora which in turn is thought to compete and defend against enteric pathogens for receptor sites on the intestinal surface or compete for intraluminal nutrients and enhance immune defences. Some probiotics have been found to be effective in treating acute diarrhea, especially when caused by rotavirus (Szymański et al., 2006; Grandy, Medina, Soria, Terán & Araya, 2010). The beneficial effects of probiotics are deemed to be strain and dose dependent and have been observed in acute, watery, and viral diarrhea but not in invasive bacterial diarrhea (Canani et al., 2007; Guandalini, 2008; Guarino et al., 2009). Alongside rehydration therapy, the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition/European Society for Paediatric Infectious Diseases suggest the use of probiotic strains with proven efficacy and no evidence of antibiotic resistant transfer (i.e. *Lactobacillus* GG and *Saccharomyces boulardii*) (Guarino, 2008).

2.2.2 Diarrhea in Neonatal Calves

2.2.2.1 Economic Impact

Diarrheal diseases are a common cause of morbidity and mortality in young calves. This complex disease develops most often within the first four weeks of life. Despite advances in research and prevention/control programs including the increasing use of Salmonella, E. coli, and clostridia vaccines, diarrhea accounts for more than 50 percent of unweaned heifer deaths (Berchtold, 2009; USDA, 2008). One-third of surveyed beef-cow operators in the United States strongly agreed or agreed that calf diarrhea has an economic impact on their operations (USDA, 2010). Diarrhea can have an economic impact on both the beef and dairy industries directly through financial losses from the loss of calves, cost of treatment, and labour needed to deliver such treatments (Barrington et al., 2002; Gunn et al., 2009; Walker et al., 1998).

2.2.2.2 Etiology

The most common cause of diarrhea in neonatal calves is due to infections of the gastrointestinal tract and usually more than one pathogen is involved. The most significant enteric pathogens are enterotoxigenic *Escherichia coli* (ETEC), *Cryptosporidium parvum*, rotavirus, and coronavirus (Foster & Smith, 2009; Gunn et al., 2009). Diarrhea due to *Salmonella* is more common in intensive calf rearing systems (Gunn et al., 2009). The cause of diarrhea also varies with the calf's age. For example, diarrhea due to ETEC is usually seen in the first three or four days of life but can also be seen in seven to fourteen days of age with rotavirus. ETEC attaches to the intestinal epithelium, colonizes and spreads through the small intestine and, once established, produces heat stable toxins resulting in secretory diarrhea (Foster & Smith, 2009). *Cryptosporidium parvum* will generally infect calves between one and four weeks of age and have clinical signs for four to fourteen days. This parasite invades the mucosa and disrupts the epithelial cells of the small intestine and leads to villus atrophy resulting in impaired nutrient digestion and absorption and malabsorptive diarrhea. Transmission is via fecal-oral by ingestion of encysted, sporulated oocysts directly from host to host and contamination of food and/or water and mechanically via flies (Gunn et al., 2009). Rotavirus is the most common cause of neonatal diarrhea in calves and affects calves five days to two weeks of age but can also occur within 24 hours of birth in colostrum-deprived calves.

2.2.2.3 Prevention and Treatment

Similar to pediatric diarrhea, the development of prevention strategies is critical in managing diarrhea. Pathogens that cause diarrhea are almost always present on the farm as many are found normally at low concentrations in the gastrointestinal tract of healthy cattle (Gunn et al., 2009). Therefore, most cattle will be exposed to these agents throughout their lifespan (Barrington et al., 2002). Whether or not neonatal calf diarrhea develops depends on many risk factors. As most infections causing diarrhea in calves occur within the first few weeks of life, ensuring adequate intake of colostrum affects the development of a calf's immune system and plays a critical role in the prevention of neonatal diarrhea. Passive immunity against enteric pathogens is associated with colostrum containing high concentrations of antibodies (Saif & Smith, 1985). For example, cows secrete anti-rotavirus antibodies in colostrum which ultimately protects against rotavirus until the antibody levels decrease in milk 48 to 72 hours

postpartum (Gunn et al., 2009). To reduce the risk of developing diarrhea, prevention strategies in neonatal calves encompasses minimizing environmental stresses and herd management conditions, reducing the exposure of pathogens, boosting immunity, and promoting biosecurity (Saif & Smith, 1985; Radostits & Acres, 1980; Schumann, Townsend, & Naylor, 1990; Gunn et al., 2009).

Regardless of the cause, neonatal calf diarrhea results in loss of water, electrolytes, and decreased milk intake which, in turn, can lead to dehydration, electrolyte imbalances, and acidosis (Smith, 2009). The most common cause of death in diarrheic neonatal calf diarrhea is due to dehydration and metabolic acidosis. D-lactate has been identified as a significant contributor to the acidosis found in diarrheic calves (Kasari & Naylor, 1984; Omole et al., 2001; Ewaschuk et al., 2004a). Production of D-lactate and D-lactic acidosis in neonatal calves is further described in Section 2.4.2.3.

To restore hydration and correct acidosis, treatment often begins with assessing dehydration status and administering fluid therapy, orally or intravenously. Oral electrolyte solutions are considered to be an economical and convenient treatment as it can be initiated on-farm and is indicated for use in diarrheic calves with dehydration, mild to moderate acidosis, those who still exhibit a suckle reflex, and at least a partially functional gastrointestinal tract (Constable, Grügerg & Carstensen, 2009). According to Smith (2009), oral electrolyte solutions should "supply sufficient sodium to normalize the extracellular fluid volume; provide agents that facilitate absorption of sodium and water from the intestine; correct the metabolic acidosis usually present in calves with diarrhea; and provide energy". Studies demonstrate benefits (i.e. provide sufficient energy and prevent weight loss) in continuing a milk diet in addition to oral electrolytes in diarrheic calves (Garthwaite, Drackley, McCoy, & Jaster, 1994; Heath, et al., 1989). Intravenous fluid therapy is indicated for use in neonatal calves when dehydration is assessed to be greater than 8% body weight or if the calf exhibits signs of severe depression, coma, unable to stand, lack of suckle reflex for more than 24 hours or a rectal temperature of less than 38°C (Berchtold, 1999).

Antimicrobials are often used to treat diarrhea in calves however due to increasing concerns surrounding antimicrobial resistance, there has been an increasing interest in the use of probiotics. In contrast to humans, few studies have investigated the use of probiotics in the treatment of calf diarrhea. Calves who received an oral dose of lactic acid-producing bacteria for

ten days proved not effective in preventing *Cryptosporidium parvum* infection (Harp et al., 1996). In a clinical setting, Ewaschuk, Zello, & Naylor (2006) tested the use of *Lactobacillus rhamnosus* GG to determine whether this probiotic would be useful for reducing the severity of diarrhea and/or reduce D-lactic acidosis. No significant differences in fecal or serum D-lactate concentrations were found between the experimental and control group and there was no significant reduction in mortality. It should, however, be noted that all calves in this study, were also clinically treated with antimicrobials which may have reduced the number of D-lactate producing bacteria. In another study, use of *Escherichial coli* strain Nissle 1917 showed a significant reduction in the frequency of calf diarrhea when compared to placebo group and, when used to treat diarrheal illness, showed a shorter period of sickness (vonBuenau et al., 2005). Further research is still required on specific strains and dosages of probiotics that are beneficial in the prevention and/or treatment of neonatal calf diarrhea, with no adverse of effects, before recommendations can be made. Recent attention has shifted to investigating the use of antimicrobial treatments, analgesic, and anti-inflammatory agents alongside fluid therapy (Constable, 2009).

2.3 Metabolic Acidosis

As arterial blood must maintain a normal pH (7.35-7.45) for proper organ function, the acid-base balance in both humans and calves is tightly regulated by the lungs, kidneys, and a system of buffers (Ayers & Warrington, 2008). Metabolic acidosis is defined as a disturbance to the acid-base balance with arterial pH < 7.35 and/or low bicarbonate concentration. This disturbance is due to an increase in blood hydrogen (H^+) concentration from the over production, or excess, of H^+ or through the loss of bicarbonate (HCO_3^-) ions (Edwards, 2008). An increase in acid may be caused by increased endogenous production (L- and D-lactic acidosis, ketoacidosis) or through ingestion of toxic substances such salicylates, methanol, ethylene glycol, or propylene glycol. Loss of bicarbonate may occur through mechanisms of the kidneys or gastrointestinal tract (Morris & Low, 2008b). Once metabolic acidosis is suspected, the serum anion gap is often calculated to diagnose the type and origin of metabolic acidosis:

$$\text{Anion Gap (AG)} = ([Na^+] + [K^+]) - ([Cl^-] + [HCO_3^-]$$

Through this calculation, metabolic acidosis is divided into either an elevated, or high, anion gap which is usually caused by an accumulation of organic acids, or normal anion gap, which can be further divided as normal hypokalemic or normal hyperkalemic (Ayers & Warrington, 2008). A review by Kraut & Madias (2006) described the variability in reported normal anion gap values, with healthy human anion gap ranges differing from one clinical laboratory to the next, from 11±2.5mEq/L to 15±2.4mEq/L to 12±4mEq/L, thus stressing the importance of clinicians to be aware of the normal range for their particular clinical laboratory. Oster, Guido, Perez, & Materson (1988) defined a normal anion gap as 8-15 mEq/L. In healthy animals, the normal anion gap also varies and ranges from 3-11 mmol/L (Ewaschuk et al., 2003).

An elevated anion gap is suggestive of an accumulation of (acidic) anions in the body such as organic acids (e.g. lactic acid, ketoacids) (Morris & Low, 2008a). An anion gap > 30 mEq is usually due to the presence of organic acidosis (i.e. ketoacidosis, lactic acidosis). Values ranging from 23-30 mEq/L are also indicative of organic acidosis but, in 30% of cases, the acid is not identifiable and values between 16 and 22 mEq/L are not thought to be very useful for diagnostic purposes (Oster et al., 1988; Kraut & Madias, 2007).

2.3.1 Metabolic Acidosis and Anion Gap in Diarrhea

Physiological changes and/or insults to the gastrointestinal tract, as in the case of diarrheal illness, can significantly impact the body's acid-base equilibrium. Metabolic acidosis in diarrhea is often described as a result of bicarbonate loss in the feces, associated with hyperchloremia, and often presents with a normal anion gap (Rose & Post, 2001). In addition to bicarbonate loss in the feces, acidosis was also thought to be due to an increase in L-lactate production as a result of hypoxic conditions such as tissue hypoperfusion or tissue hypoxemia associated with dehydration (Perez et al., 1987). In the case of moderate to severe diarrhea, a high anion gap may be present (Weizman, Houri, & Ben-Ezer Gradus, 1992; Wang, Butler, Rabbani, & Jones, 1986; Boonsiri, Tangrassameeprasert, Panthongviriyakul, & Yongvani, 2007). In a retrospective cohort chart review of 467 children admitted to a pediatric observation unit with dehydration caused by gastroenteritis, 25% of children had an anion gap greater than 14 mmol/L (mean: 16.7 mmol/L) and ranged from 1-28 mmol/L (Mallory, Kadiesh, Zebrack, & Nelson, 2006). Elevated anion gaps have also be been observed in diarrheic neonatal animals (Omole et al., 2001; Ewaschuk, Naylor, & Zello, 2003) and humans with short bowel syndrome

(Bongaerts et al., 2000). In clinical cases of metabolic acidosis in humans with short bowel syndrome, anion gaps ranging from 20-32 mmol/L have been reported (Coronado, Opal, & Yoburn, 1995; Htyte et al., 2011; James, Black, Kuper, & Saibil, 2010; Munakata et al., 2009; Puwanant et al, 2005; Zhang, ZW Jiang, J Jiang, Cao, & Li, 2003). The mean anion gap in diarrheic calves, when compared to healthy calves, may increase by more than 14 mmol/L and ranges of 7.0-37.5 mmol/L have been reported (Omole et al., 2001; Ewaschuk et al., 2004a). The anion gap significantly correlates with serum D- and total DL-lactate concentrations but not with serum L-lactate concentrations as previously thought (Ewaschuk et al., 2003).

Regardless of the type of acidosis that occurs, left untreated, acidosis can lead to serious complications including death. Acidemia (pH < 7.1) may lead to changes in the structures and functions of proteins and enzymes which can impose negative effects on the body such as inhibiting cardiac output; hypotension, hypoperfusion of organs; hyperkalemia; hypercalcemia; hypercalciuria; and increased protein catabolism (Ayers & Warrington, 2008).

2.4 Lactate

Lactate (2-hydroxypropanoate) is a chiral, hydroxycarboxylic acid that exists as two stereoisomers: D-lactate and L-lactate. These isomers differ only in the position of the alpha-hydroxy group (Figure 2.1).

Figure 2.1 Lactate stereoisomers (Horton, 2002.)

Both L- and D-lactate can be produced endogenously by humans and calves and is further described in Sections 2.4.1 and 2.4.2.1, respectively. Exogenous sources of lactate are found in fermented foods such as yogurt, kefir, pickles, sauerkraut, some cheeses, meat, red wine, and is

used as a food additive. When these foods are consumed by healthy individuals the lactate contained in these foods does not appear to contribute to clinical cases of acidosis as it is likely amounts consumed are not large enough to exceed an individual's capacity to metabolize it (de Vrese & Barth, 1991; Halperin & Kamel, 1996; Haschke-Becher et al., 2008)

2.4.1 L-Lactate and L-Lactic Acidosis

Under physiological aerobic conditions pyruvate, the last product of glycolysis, enters the mitochondria and is oxidized to acetyl CoA for entry into Krebs cycle for ATP production (Horton, 2002). When the oxygen supply is insufficient, tissues produce lactate by anaerobic glycolysis (Figure 2.2). In this pathway, pyruvate is reduced by NADH to produce L-lactate. This reaction is catalyzed by an enzyme, lactate dehydrogenase (LDH), located in the cell cytosol (Horton, 2002; Luft, 2001). The production of L-lactate by this pathway makes NAD^+ available and allows glycolysis to continue under anaerobic conditions. This reaction is reversible and, once oxygen becomes available, excess lactate will be metabolized back to pyruvate (De Backer, 2003). Blood lactate is metabolized by the liver and kidney. In muscle cells, via the Cori cycle, lactate produced by anaerobic glycolysis travels to the liver where, through gluconeogenesis, it is converted glucose and returned to the muscles (Vernon & LeTourneau, 2010). Over the course of the day, approximately 1500 mmol/L of lactate can be produced (Levy, 2006). In healthy humans and calves, blood lactate levels will range between 1-2 mmol/L. This blood lactate concentration is considered to be predominately in the form of L-lactate (Luft, 2001; Vernon & Letourneau, 2010).

In the literature, the term "lactic acidosis" refers to the more commonly known condition of L- lactic acidosis characterized by hyperlactemia, > 5 mmol/L, and pH < 7.35 (Luft, 2001). L-lactic acidosis is most often observed in physiological conditions where oxygen delivery to the tissues is inadequate resulting in tissue hypoxia and production of excess L-lactate (Vernon & LeTourneau, 2010). L-Lactic acidosis can also occur as a result of impaired L-lactate metabolism in which excess lactate can result from an overproduction and/or decreased clearance due to underlying disease (renal and/or hepatic failure, diabetes mellitus, malignancy, systemic inflammatory response syndrome and HIV), effects of drugs, and inborn errors of metabolism (Vernon & LeTourneau, 2010).

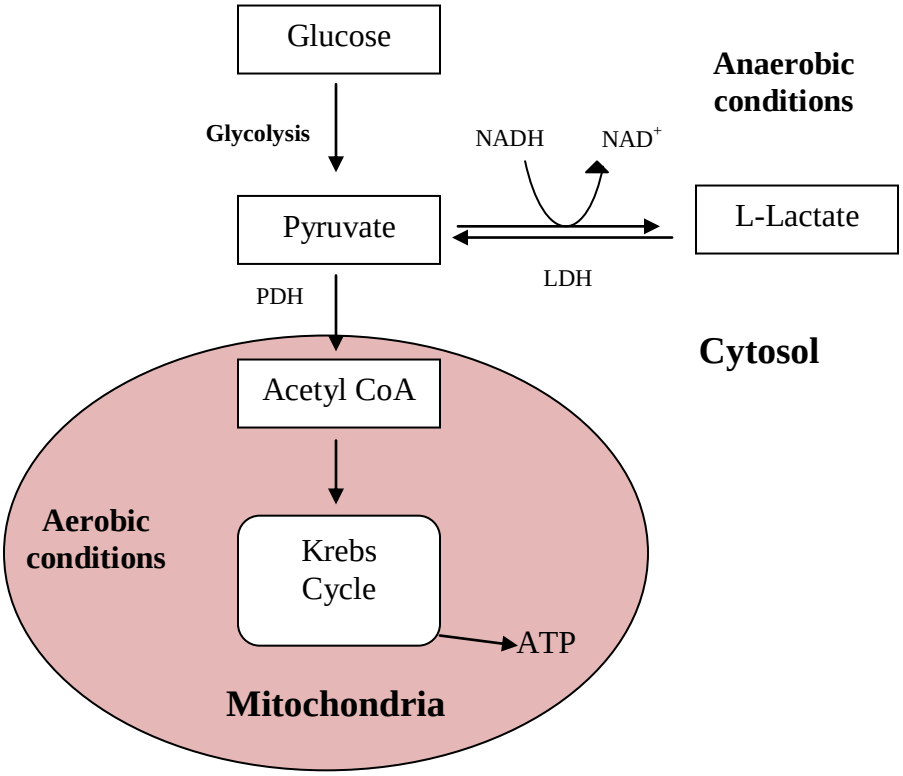

Figure 2.2 L-Lactate production. In normal aerobic conditions, pyruvate is oxidized in the citric acid cycle. In anaerobic conditions pyruvate will be reduced to produce L-lactate. PDH, pyruvate dehydrogenase complex; LDH, lactate dehydrogenase. Adapted from Horton, 2002.

2.4.2 D-Lactate and D-Lactic Acidosis

2.4.2.1 D-Lactate Production

Although L-lactate is the predominant form of lactate found in blood, small amounts of D-lactate are produced endogenously in humans and calves through the glyoxalase pathway in the cytosol of all cells (McLellan & Thornalley, 1989; Thornalley, 1993). The glyoxylase system detoxifies and catalyses the conversion of methylglyoxal, a reactive glycating agent, formed when carbohydrates, lipids, and amino acids are metabolized (Figure 2.3). Through this system, methylglyoxal is metabolized to D-lactate via the intermediate S-D-lactoylglutathione, two enzymes: glyoxylase 1 (Glo1) and glyoxylase 2 (Glo2), and reduced glutathione (Kalapos, 2008; Xue, Rabbani & Thornalley, 2011; McLellan & Thornalley, 1989). In healthy physiological conditions, the amounts of D-lactate produced by this pathway are thought to be minimal.

D-Lactate is also normally produced in the gastrointestinal tract of humans and animals through the bacterial fermentation of carbohydrates (i.e. fibre and incompletely digested starch, lactose, and proteins) (Duncan, Louis, & Flint, 2004; Halperin & Kamel, 1996). A heterogeneous group of lactic acid producing bacteria is found in the gastrointestinal tract which can produce D-lactate, L-lactate, or both including gram-positive *Streptococcus, Pediococcus, Lactobacillus, Leuconostoc, Bifidobacterium,* and *Eubacterium* spp. (Duncan et al., 2004; Hove & Mortensen, 1995). Some bacteria also possess DL-lactate racemase, an enzyme that changes one lactate isomer to the other (Hove & Mortensen, 1995). In healthy humans, colonic lactate is further metabolized to short chain fatty acids. A study by Bourriaud et al (2004) suggests butyrate is the major net product of lactate conversions. Total DL-lactate concentrations in human feces are typically less than 2-3 mmol/L (Hove & Mortensen, 1995; Mortensen, Hove, Clausen, & Holtug, 1991). In healthy neonatal animals, a wide variation of fecal D-lactate concentrations have been reported in the literature ranging from 1.2-24.4 mmol/L (Omole et al., 2001; Ewaschuk et al., 2004a; Shimomura & Sato, 2006; Sato & Koiwa, 2008). In calves, Sato & Koiwa (2008) observed fecal lactate concentrations to be at its highest at one week of age and progressively decreases with advancing age suggesting newborn calves depend on the colon rather than the rumen for gut fermentation as they obtain most of their nutrients from liquid milk.

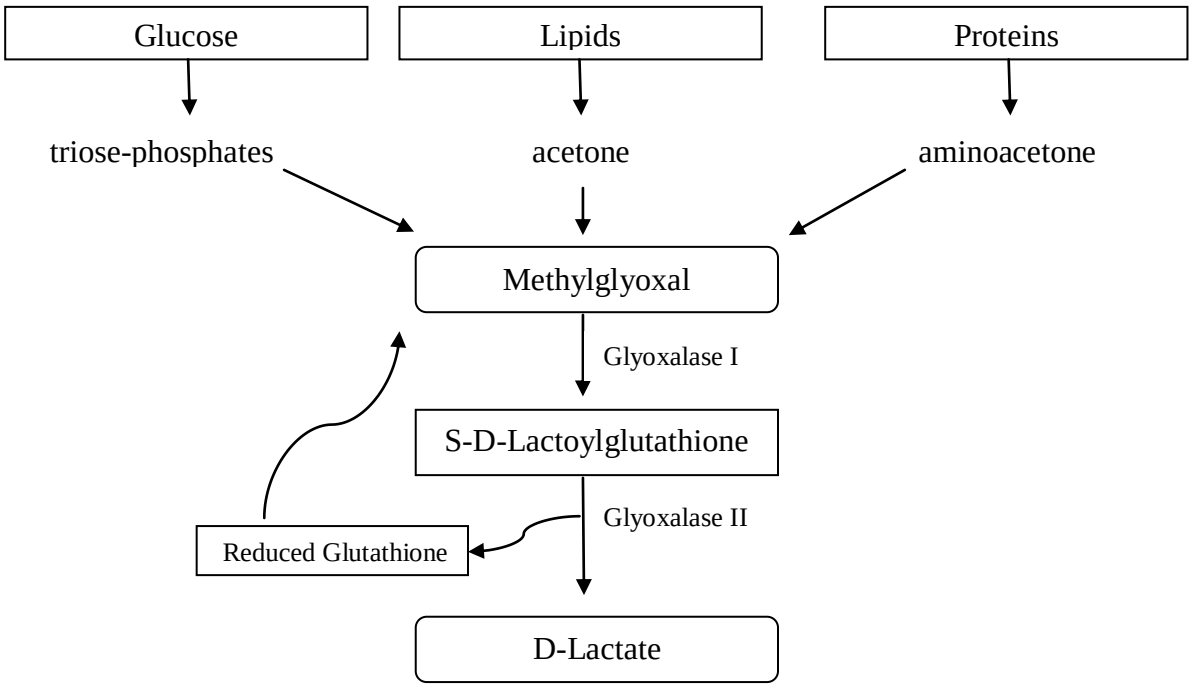

Figure 2.3 Endogenous D-lactate production. The glyoxalase pathway produces D-lactate via the intermediate S-D-lactoglutathione. It uses two enzymes, glyoxalase I and glyoxalase II, and a catalytic amount of reduced glutathione. Adapted from (Ewaschuk et al., 2005; Thornalley, 1993)

Despite the D-lactic acid producing bacteria in the gastrointestinal tract and endogenous production via methylglyoxal pathway, D-lactate does not appear to pose a threat to the acid-base balance in healthy humans and calves. Only very small amounts, usually unquantifiable and undetectable by routine laboratory tests, of D-lactate are present in the blood of healthy humans and calves and accounts for about 1-5% of L-lactate concentrations (McLellan, Phillips, & Thornalley, 1992). In the literature, D-lactate concentrations in healthy adult humans have been reported to be less than 0.1 mmol/L (Ewaschuk et al., 2005; Hove & Mortensen, 1995; McLellan, Phillips, & Thornalley, 1992; de Vrese & Barth, 1991; Herrera, Morris, Johnston & Griffiths, 2008). Few studies have examined blood D-lactate reference ranges in children. Connolly, Abrahamsson & Björksten (2005) reported D-lactate concentrations similar to that of

adults ranging from 0.03-0.055 mmol/L while Herrera et al. (2008) reported a mean plasma D-lactate of 0.011 mmol/L. In healthy neonatal animals, blood D-lactate concentrations of 0.20-1.40 mmol/L have been reported (Abeysekara, Naylor, Wassef, Isak, & Zello, 2007; Ewaschuk et al., 2003; Ewaschuk et al., 2004a; Lorenz, 2004).

2.4.2.2 Transport and Metabolism

Once D- and/or L-lactate are produced in the gastrointestinal tract they can be absorbed into the blood via the large intestinal epithelium. Transport of lactate is achieved via proton-linked monocarboxylate transporters (MCTs) which are found in almost all tissues including skeletal muscle, heart, brain, liver and intestinal epithelial cells (Halestrap & Price, 1999). MCTs have been identified on the apical and basalateral membranes in the colon (Gill et al., 2005). More specifically, MCT-1 has been identified on the basalateral membranes of colonic enterocytes and is thought to be the major transport mechanism by which lactate, pyruvate, and short chain fatty acids enter the systemic circulation from the gastrointestinal tract (Lam, Felmlee, & Morris, 2009; Iwanaga, Takebe, Kato, Karaki, & Kuwahara, 2006; Gill et al., 2005). Once in the blood, D-lactate was initially thought to be metabolized very slowly as humans did not appear to have an enzyme specifically for the D-isomer, D-lactate dehydrogenase (D-LDH) (Stolberg et al., 1982). To date, a large body of evidence now supports the ability of humans to effectively metabolize D-lactate (Connor, Woods, & Ledingham, 1983; Oh et al., 1985; Hove & Mortensen, 1995). After administering human volunteers with 6.4 mmol/kg of racemic DL-lactic acid, de Vrese, Koppenhoefer, & Barth (1990) reported a D-lactate half life of 28.6 ± 4.3 minutes with less than 2% of D-lactate excreted in the urine during 24 hours following intake with no significant decrease in blood pH.

In mammals, the enzyme, D-α-hydroxy-acid dehydrogenase, is thought to be responsible for metabolizing D-lactate to pyruvate and is found to be highly active in the kidney and liver (Uribarri et al., 1998). This enzyme is non-specific as it contains substrates for other D-2-hydroxy acids and metabolizes D-lactate to pyruvate at approximately one-fourth the rate that L-lactate dehydrogenase metabolizes L-lactate (Ewaschuk et al., 2005; Tubbs, 1965). More recently, hepatic mitochondrial putative D-lactate dehydrogenase has been isolated and is similar to the D-LDH found in lower organisms (de Bari, Atlante, Guaragnella, Principato, & Passarella, 2002; Flick & Konieczny, 2002). From the blood, lactate enters the liver via proton-dependent

MCTs, is transported into the mitochondria and oxidized by putative D-LDH to pyruvate (Poole & Halestrap, 1993; de Bari et al., 2002).

In the kidney, L- and D-lactate mutually interfere with their renal re-absorption (de Vrese et al., 1990; Oh et al., 1985). Oh et al. (1985) found complete re-absorption of L-lactate when blood D-lactate concentrations were less than 3 meq/L; but when D-lactate concentrations exceeded 3 meq/L, re-absorption of L-lactate was impaired resulting in increased urinary excretion of this isomer. When compared to the interference of L-lactate with D-lactate, re-absorption of L-lactate is more efficient (Oh et al., 1985). The mechanisms behind this are not yet clear but it has been proposed that D- and L-lactate use the same co-transport mechanism with sodium but with a greater affinity for the L-lactate (Oh et al., 1985). Small amounts of D-lactate are excreted in the urine and, in healthy adults; urinary D-lactate concentrations have been reported to be < 0.7 mmol/L (Hove & Mortensen, 1995). In healthy infants and children, urinary excretion of D-lactate is found to be highest during the first year of life ranging from 6.4-39.9 mmol/mol creatinine which then decreases and stabilizes from 2.5 to 4 years of age (Haschke-Becher, Baumgartner, & Bachmann, 2000). D-lactate is also excreted in the urine of healthy neonatal calves with concentrations ranging from 0.5-7.4 mmol/L (Ewaschuk et al., 2004a).

2.4.2.3 D-Lactic Acidosis in Humans

In humans, D-lactic acidosis has been defined as "metabolic acidosis accompanied by increase in serum D-lactate concentration in excess of 3 mmol/L" (Uribarri et al., 1998). Human cases of D-lactic acid were first described in adults and children in 1979 and 1980, respectively (Uribarri et al., 1998; Oh et.al, 1979; Schoorel, Biesberts, Blom & van Geldern, 1980). This type of acidosis is considered to be rare and the majority of cases are reported as a clinical complication of short-bowel syndrome (Vella & Farrugia, 1998; Hove & Mortensen, 1985; Al Chekakie et al., 2004; Bongaerts et al., 1995). More recently, Grünert et al. (2010), reported D-lactic acidosis in a child with carbohydrate malabsorption syndrome of an unknown cause. Propylene glycol ingestion has also been reported as a cause of D-lactic acidosis (Christopher, Eckfeldt, & Eaton, 1990; Jorens et al., 2004).

D-Lactic acidosis may or may not include neurological symptoms. Clinical signs and symptoms appear to be non-specific and vary in duration. A correlation has not been shown

between D-lactate levels and the severity of D-lactic acidosis symptoms which include slurred speech; blurred vision; confusion; disorientation; delirium; dizziness; ataxia; lethargy; lack of concentration; somnolence; hallucinations; weakness; unsteady gait; irritability; nystagmus; and even abusive/hostile behaviour (Uribarri, et al., 1998; Stolberg et al., 1982; Al Chekakie et al., 2004). D-lactic acidosis has also been associated with episodes of encephalopathy and recurrent encephalopathy (Htyte et al., 2011; Grünert et al., 2010; Munakata et al., 2009; Uribarri et al., 1998; Abeysekara et al., 2007). Physiological conditions required for the development of D-lactic acidosis have been proposed by Uribarri et al. (1998) and include: carbohydrate malabsorption, d-lactic producing colonic bacteria, consumption of large amounts of carbohydrates, reduced colonic motility, and/or impaired D-lactate metabolism. These conditions are present, most often, in SBS and its pathophysiology is further described in Section 2.4.2.3.1. Interestingly, these conditions are similar to those found in acute diarrhea, however, little research has examined whether or not D-lactic acidosis in humans with acute diarrhea in absence of SBS. Sack et al. (1982) investigated acidosis and carbohydrate malabsorption in infants with rotavirus diarrhea and found little detectable fecal bicarbonate, but the origin of the acidosis was never identified.

D-Lactic acidosis occurs as a result of increased D-lactate production caused by excessive fermentation of carbohydrate in the gastrointestinal tract and accumulation in the blood. High fecal D-lactate concentrations provide evidence for the intestinal origin of D-lactic acidosis rather than overproduction via methylglyoxal pathway as described in Section 2.4.2.1 (Hove & Mortensen, 1995). Stolberg et al. (1982) describe fecal flora in D-lactic acidosis as “markedly abnormal” with the majority being gram-positive anaerobes known to produce D-lactate including lactobacillus, eubacterium, and bifidobacterium species. Administration of oral antibiotics, vancomycin, resulted in the return of “normal” stool flora and less D-lactic produced by cultured bacteria (Stolberg et al., 1982).

2.4.2.3.1 Short Bowel Syndrome

Short bowel syndrome (SBS) has been defined as inadequate functional bowel to support nutrient and fluid requirements. In terms of bowel length, 100–120 centimetres (cm) of small bowel without a colon, 50 cm of small bowel with a colon, or a 70%–75% loss of small bowel may result in symptoms associated with SBS (Parrish, 2005). As a result of this loss, the ability

to digest and absorb nutrients is impaired and diarrhea, dehydration and nutrient deficiencies are often documented in SBS (Puwanant, Mo-Suwan, & Patrapinyokul, 2005; Bongaerts et al., 1997; Parrish, 2005).

The pathogenesis of D-lactic acidosis in SBS is well described in the literature. The shortening, or removal, of the small intestine may result in the malabsorption of fluid and nutrients. A larger than normal amount of substrate (i.e. carbohydrates) delivered to the colon increases bacterial fermentation producing an unusually high amount of organic acids (short chain fatty acids, D- and L-lactic acid). The accumulation of these organic acid results in decreased lumen pH which, in turn, leads to a loss in the normally predominant bacterial flora, bacteroides species, and favours the growth of acid-resistant, gram positive, D- and L-lactate producing anaerobic bacteria (i.e. *Lactobacillus)* (Al Chekakie et al., 2004; Uribarri, et al., 1998). Larger than normal amounts of D- and L-lactate acid are then produced in the gastrointestinal tract and absorbed into the systemic circulation. Once absorbed, L-lactate is more effectively metabolized which leads to elevated blood D-lactate and D-lactic acidosis (Hove & Mortensen, 1995; Ewaschuk et al., 2005; Petersen, 2005).

Neurological symptoms associated with D-lactic acidosis may resolve with or without treatment (Al Chekakie et al., 2004). Treatment of D-lactic acidosis usually begins with fluid and electrolyte replacement with sodium bicarbonate to correct the acidosis. To reduce the formation of D-lactate in the gastrointestinal tract, treatment may also include the use of antibiotics and restriction of carbohydrates to reduce the quantity of substrate for bacterial fermentation (Uchida et al., 2004; Al Chekakie et al., 2004).

2.4.2.4 D-Lactic Acidosis in Neonatal Animals with Diarrhea

Although a rare condition in humans, D-lactic acidosis is well known and described in veterinarian medicine as a consequence of grain overfeeding in adult cattle and ruminal drinking in calves (Gentile et al., 2009; Dunlop & Hammond, 1965; Ewaschuk et al., 2004a). Initially, in neonatal calf diarrhea, metabolic acidosis was thought to result from the loss of bicarbonate in the feces and accumulation of L-lactate in the blood as a result of hypoxic conditions associated with dehydration. In calves, Kasari & Naylor (1984) observed that acidosis can occur in the absence of dehydration, which suggested there may be a different origin of metabolic acidosis than previously thought. Omole et al. (2001) examined the spectrum of organic acids in neonatal

diarrheic calves with diarrhea. This study found that diarrheic calves had significantly higher serum concentrations of pyruvic acid, acetic acid, and D- and L-lactic acids. Results of this study also revealed that acidosis in diarrheic calves is due, in part, to D-lactic acid accumulation as the serum concentrations of D- and L-lactate were elevated, in comparison to healthy calves, and accounted for 64% anion gap increase. Further studies investigating D-lactate in neonatal calves (Abeysekara et al., 2007; Ewaschuk et al., 2003; Ewaschuk, et al., 2004a; Lorenz, 2004; Lorenz & Lorch, 2009), lambs (Abeysekara, 2009), and goat kids (Bleul et al., 2006) with diarrhea support these findings.

Similarly to humans, high concentrations of fecal D-lactate suggest that the gastrointestinal tract is the primary source of D-lactate production in D-lactic acidosis in neonatal animals with diarrhea (Ewaschuk et al., 2004a; Omole et al., 2001). The process in which high amounts of D-lactate are produced is thought to be similar to that in SBS but instead of carbohydrate malabsorption caused by a shortened gastrointestinal tract, malabsorption results from the villous atrophy caused by diarrheic enteric pathogens (Ewaschuk et al., 2005; Ewaschuk et al., 2006). Recent research in diarrheic lambs and calves also suggest that high levels of blood D-lactate appear after high levels of D-lactate are produced in the gastrointestinal tract. In other words, a potential gastrointestinal threshold may exist for the absorption of D-lactate into the blood in diarrheic neonatal animals. Using a biphasic (breakpoint) regression analysis the fecal threshold for D-lactate absorption in lambs and calves was reported to be 10.2 and 8.8 mmol/L, respectively (Abeysekara, 2009; Zello et al., 2009).

D-Lactic acidosis in neonatal calves with diarrhea has been associated with impaired neurological dysfunction including disturbance of the palpebral and menace reflexes; depression with weakness and ataxia; impaired posture and behaviour; and coma (Kasari & Naylor, 1984; Lorenz, 2004). Abeysekara et al (2007) demonstrated that high infusions of D-lactate to calves can cause acute neurocardiac toxicity; however, the mechanism is not yet clearly understood. Sodium bicarbonate is usually the treatment of choice for neonatal calves with diarrhea and acidosis. Once acidosis is corrected and body fluid volume is restored, D-lactate concentrations are reported to decrease (Kasari & Naylor, 1985; Lorenz & Vogt, 2006). As briefly described in Section 2.2.2.3, Ewaschuk et al. (2006) investigated whether a probiotic, *Lactobacillus rhamnosus* GG would reduce the severity of diarrheal and/or D-lactic acidosis, however, the

potential benefits were not clear and further research in the use of probiotics in D-lactic acidosis is required before recommendations can be made.

2.5 Methodology for the Analysis of D- and L-Lactate

In the clinical setting, analytical methods do not routinely measure D- and L-lactate concentrations separately (i.e. blood gas and portable lactate analyzers) and assays for specific D- and L-lactate isomers are generally not available (Talasniemi, Pennanen, Savolainen, Niskanene & Liesivuori, 2008). Measuring total lactate in biological fluids provides limited information on the origin of lactate production. Analyzing biological fluids, such as blood and feces, for each lactate isomer can provide important information on the origin of the acidosis and clinical diagnosis (i.e. D-lactic acidosis) in order to initiate appropriate and effective medical treatment. Several methods are available for measuring D- and L-lactate in biological materials including enzymatic assays, high performance liquid chromatography (HPLC), HPLC coupled with tandem mass spectrophotometer (HPLC/MS/MS), gas chromatography coupled with mass spectrophotometer (GC-MS) and capillary electrophoreseis (CE) (Herrera et al., 2008; Ewaschuk, Naylor, Barabash, & Zello, 2004; Ewaschuk, Zello, Naylor, & Brocks, 2002; Henry, Marmy Conus, Steenhout, Béguin, & Boulat, 2011). In regards to D-lactate analysis, many of these methods are used to determine, or diagnose, D-lactic acidosis (> 3 mmol/L). The enzymatic assay method is described in further detail below.

2.5.1 Enzymatic Methods

Enzymatic assays are commonly used to determine D- and L-lactate concentrations in biological fluids using ultraviolet or fluorometric detection (Talasniemi et al, 2008; Herrera et al., 2008; Haschke-Becher et al., 2000; McLellan, Phillips & Thornalley, 1992). For quantification of L-lactate, the enzymatic method requires two coupled reactions and is based on the oxidation of lactate to pyruvate by lactate dehydrogenase (LDH) with reduction of NAD+ to NADH (Figure 2.4, part 1). The second reaction removes pyruvate to shift the equilibrium towards production of NADH. Pyruvate is converted to L-alanine and 2-oxoglutarate by reacting with L-glutamate and catalyzed by the enzyme, L-alanine aminotransferase (Figure 2.4, part 2).

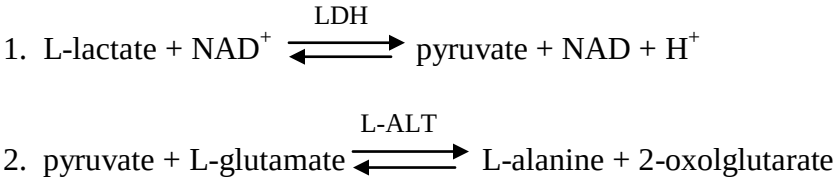

Figure 2.4 Chemical reactions for the measurement of L-lactate

The amount of NADH produced in the reaction using spectrophotometry at absorbance of 340 nm is directly proportional to the amount of L-lactate in the sample (Ewaschuk et al., 2002; Herrera et al., 2008; Talasniemi et al., 2008). D-lactate is measured using the same reactions. In the first reaction (Figure 2.4, part 1), D-lactate becomes the substrate instead of L-lactate and the reaction is catalyzed by D-lactate dehydrogenase (D-LDH) instead of lactate dehydrogenase. A disadvantage of enzymatic methods when measuring D-lactate is the non-specific transformation of NAD^+ to NADH due to oxido-reductases in the samples, which can lead to overestimations of D-lactate concentrations (Herrera et al., 2008). In response to this effect, modifications to enzymatic methods have been described including sample deproteinization with perchloric acid to make the sample protein free (Brandt, Siegel, Waters & Bloch, 1980; McLellan et al., 1992). Herrera et al. (2008) validated an enzymatic method, which uses a sample blank correction in which absorption of a sample blank is subtracted from total absorbance. In developing this method, Herrera et al. (2008) also identified that elevated lactate dehydrogenase activity (>1500 IU/L) and L-lactate concentrations may result in underestimation of D-lactate concentrations, due to the differences in L-LDH and D-LDH affinity for NAD^+. Sample ultrafiltration procedures are required to remove such interference (Herrera et al., 2008; Marti et al., 1997). More recently, Nielsen, Pedersen, Lindholt, Mortensen & Erlandsen (2011) pre-treated samples with sodium hydroxide (NaOH) to inactivate the L-LDH activity in plasma samples.

The advantage of using the enzymatic method is that many commercial assay kits are readily available (Talasniemi et al. 2008; Herrera et al., 2008; Nielsen, 2011). These kits are, for the most part, simple to use and require minimal laboratory equipment (i.e. spectrophotometer) and can be used in areas where access to more advanced laboratory analysis equipment (i.e. HPLC) may not be available. The quantitation limit for the enzymatic method has also been

shown to be lower in comparison to other analytical methods. For example, an HPLC method developed by Ewaschuk et al. (2004b) reported a quantitation limit for D- and L-lactate in calf feces, rumen fluid and urine of 1.0 mmol/L. In serum, the validated lower limit of quantitation for D-lactate was 0.5 mmol/L (Omole et al. 2001). Using an enzymatic assay method, Nielsen, Pedersen, Lindholt, Mortensen, & Erlandsen (2011) reported a D-lactate limit of quantification of 0.010 mmol/L. In addition to quantifying D-lactate in acidosis, the ability to quantify lower concentrations of D-lactate may prove to be quite beneficial for the investigation of subclinical elevations of blood D-lactate as observed in such clinical cases of diabetes mellitus (McLellan, Thornalley, Benn, & Sonksen, 1994).

2.6 Summary

Diarrhea continues to be a leading cause of morbidity and mortality in both children and neonatal calves. Despite the cause, diarrhea can result in clinical consequences including electrolyte disturbances, dehydration, and metabolic acidosis. Metabolic acidosis may arise from excess, or overproduction, of blood H^+ through increased endogenous production of acids including lactic acid or through the loss of bicarbonate via kidneys or gastrointestinal tract (Edwards, 2007). In diarrhea, it was initially thought that acidosis arose from the loss of bicarbonate in the feces and increased production of L-lactate. However, research has shown that in diarrheic neonatal calves D-Lactate is as a significant contributor to metabolic acidosis (Kasari & Naylor, 1984; Omole, et al., 2001; Ewaschuk et al., 2004a).

D-Lactic acidosis can result in impaired neurological dysfunction and, in severe cases, lead to encephalopathy and coma (Kasari & Naylor, 1984; Lorenz, 2004; Uribarri et al., 1998). As D-lactic acidosis differs from that of L-lactic acidosis, it is important to be able to quantify both D- and L-lactate concentrations in biological fluids for appropriate diagnosis and clinical treatment. Upon collection of biological samples, pre-analytical conditions such as blood processing and storage may influence the stability of various solutes in blood samples following collection. Most research examines the stability of total lactate, D- and L-, and little is known about the stability of each lactate isomer. Further research is warranted to examine the stability of D- and L-lactate concentrations in calf blood samples to ensure a reliable measure of D-lactate.

In humans, D-lactic acidosis is thought to be rare and the majority of cases are reported

as a complication of SBS (Vella & Farrugia, 1998; Hove & Mortensen, 1985; Al Chekakie et al., 2004; Bongaerts et al., 1995**).** The conditions reported to support the overproduction of D-lactate in SBS (i.e. carbohydrate malabsorption, D-lactic acid producing bacteria and reduced colonic motility) are similar to that found in acute diarrhea. Further research examining whether elevated D-lactate concentrations and/or D-lactic acidosis occurs in children with acute diarrhea is needed.

A high concentration of fecal D-lactate in humans with SBS and diarrheic animals suggests that the gastrointestinal tract is the site of D-lactate production in D-lactic acidosis (Ewaschuk et al., 2004a; Omole et al., 2001; Uribarri et al., 1998). A study suggests that blood D-lactate concentrations increase only after high levels are produced in the gastrointestinal tract and a fecal threshold was proposed (Abeysekara, 2009; Zello et al., 2009). As D-lactate concentrations below 0.05 mmol/L were considered to be not quantified and an arbitrary value of 0.04 mmol/L was used in the statistical calculations, further research examining a wide range of diarrhea severity and use of an analytical method to detect D-lactate levels below 0.5 mmol/L (i.e. enzymatic assay method as described in Section 2.5.1) is warranted to confirm this fecal threshold in the gastrointestinal tract at which D-lactate enters the systemic circulation in neonatal calves with diarrhea.

CHAPTER THREE
THE EFFECT OF PREANALYTICAL PROCESSING AND STORAGE ON BLOOD D- AND L-LACTATE CONCENTRATIONS

3.1 Introduction

Metabolic acidosis is defined as a disturbance to the body's acid-base balance (pH < 7.35) and, if left untreated, can lead to serious clinical complications in both humans and animals (Edwards, 2007). Identifying the underlying cause and major contributing acid is important for initiating appropriate clinical treatment. Lactic acidosis is one type of elevated anion gap acidosis resulting from an increased production and/or decreased clearance of lactate (Morris & Low, 2008b). Lactate is a hydroxycarboxylic acid and exists as two stereoisomers, D-lactate and L-lactate (Horton, 2002). Under healthy physiological conditions, blood lactate concentrations are predominately in the form of L-lactate whereas D-lactate is present in very low concentrations. The term "lactic acidosis" generally refers to the more commonly known condition of L-lactic acidosis observed in physiological conditions where oxygen delivery is inadequate leading to tissue hypoxia and excess production of L-lactate (Vernon & LeTourneau, 2010). D-Lactate has been identified as a significant contributor to metabolic acidosis in diarrheic calves (Kasari & Naylor, 1984; Omole, et al., 2001; Ewaschuk et al., 2004a) and elevated serum D-lactate concentrations and D-lactic acidosis (serum D-lactate > 3 mmol/L) has been reported in several species including diarrheic lambs (Abeysekara, 2009; Lorenz & Lorch, 2009) goat kids (Bleul et al., 2006), and humans with short bowel syndrome (Uribarri et al., 1998). The clinical presentation of D-lactic acidosis differs from that of L-lactic acidosis. Altered mental and physical states such as weakness, ataxia, impaired posture and behaviour have been reported and, in severe cases, acute and recurrent encephalopathy and coma (Kasari & Naylor, 1984; Kasari & Naylor, 1986; Lorenz, 2004; Lorenz et al., 2005; Forsyth, Moulden & Hull, 1991; Grünert et al., 2010; Htyte et al., 2011; Jorens et al., 2004).

Changes in blood constituent concentrations may occur after specimen collection. Serum or plasma samples that are not promptly separated from red blood cells following collection may contain artifactually high levels of lactate since red blood cells (RBC) can continue to uptake and metabolize glucose *in vitro* and produce both isomers of lactate. In general, it is recommended that serum and plasma for biochemical analysis be separated from

cells as soon as possible and held no more than 4 hours at 4°C prior to processing to prevent ongoing cellular metabolism and transport of analytes between plasma or serum and cellular components (Tuck et al., 2009). However, outside the clinical and/or laboratory setting, this may not always be possible. Samples collected in the field may require temporary storage until transported to a biochemistry laboratory for analysis. This may result in a delay of blood centrifugation and exposure of the sample to variable temperatures before analysis. Similar situations may also occur when blood samples are collected from remote areas (i.e. developing countries) where access to appropriate storage units, a centrifuge, and/or biochemistry laboratories may be limited. Storage and processing delays may also occur in clinical chemistry laboratories, which handle large numbers of samples requiring biochemical analysis. Research has shown that even a fifteen minute delay in processing human whole blood samples at room temperature, or storage for 1 hour at 4°C, can result in a significant overestimation of initial lactate levels (Calatayud & Tenías, 2003). Significant changes in lactate concentration over time have also been documented in human plasma and serum left in prolonged contact with blood cells (Boyanton & Blick, 2002).

Previous research on lactate stability has only investigated the change in lactate concentrations. Less is known about the effects of specimen collection and storage on the blood levels of specific lactate isomers, D- and L-lactate. As D-lactic acidosis is a unique type of metabolic acidosis with clinical symptoms differing from that of L-lactic acidosis (i.e. neurological dysfunctions) it is important to be able to quantify both D- and L-lactate concentrations in blood for proper clinical diagnosis and treatment to prevent acidosis and reduce overall acidosis associated morbidity and mortality. As blood is not always collected in a laboratory and/or clinical setting, the effects of processing and storage need to be determined on both isomers to ensure a reliable measurement of D- and L-lactate concentrations. The objectives of this study were to examine changes of D- and L-lactate concentrations over time in serum and plasma stored (a) in contact with blood cells and (b) after separation via centrifugation.

3.2 Materials and Methods

3.2.1 Subjects

As D-lactic acidosis is a well known complication of neonatal calf diarrhea and biological samples from these animals are often collected in the field, calves were chosen as the subjects in this study. Blood samples were obtained from eleven healthy Holstein breed calves, seven to fifteen days of age, from the Dairy Barn of the Department of Animal and Poultry Sciences, College of Agriculture and Bioresources, University of Saskatchewan. From five of the eleven calves, both serum and plasma were prepared from the same blood collection. Six of the eleven calves were used for preparation of either serum (3 calves) or plasma samples (3 calves). This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use (Appendix A).

3.2.2 Sample Collection and Analysis

Blood (60-120 mL), was collected by jugular venipuncture in manually restrained calves. Samples were processed as shown in figure 3.1. Immediately following collection (time 0), half of the blood (30-60 mL) was spiked with DL-lactic acid to achieve a final concentration of 3 mmol/L for each isomer (3 mmol/L D-lactate and 3 mmol/L L-lactate; from now on referred to as "untreated" samples). The blood samples were spiked to mimic those levels seen in calves with acidosis. The remaining blood was left untreated (from now on referred to as "untreated" samples).

For each individual calf, half of the spiked and untreated blood samples, respectively, were distributed into tubes containing lithium heparin (Vacutainer® PST™ Tubes; BD) for plasma collection and the remaining blood was distributed into Vacutainer® serum tubes with a proprietary thrombin-based medical clotting agent and a polymer gel (Vacutainer® Rapid Serum Tubes; BD or Micro Tube; SARSTEDT) for serum collection. Tubes were allowed to stand for 30 minutes at room temperature. Half of the serum and plasma tubes were then centrifuged for separation of plasma or serum and the remaining half were stored in contact with blood cells at 4°C.

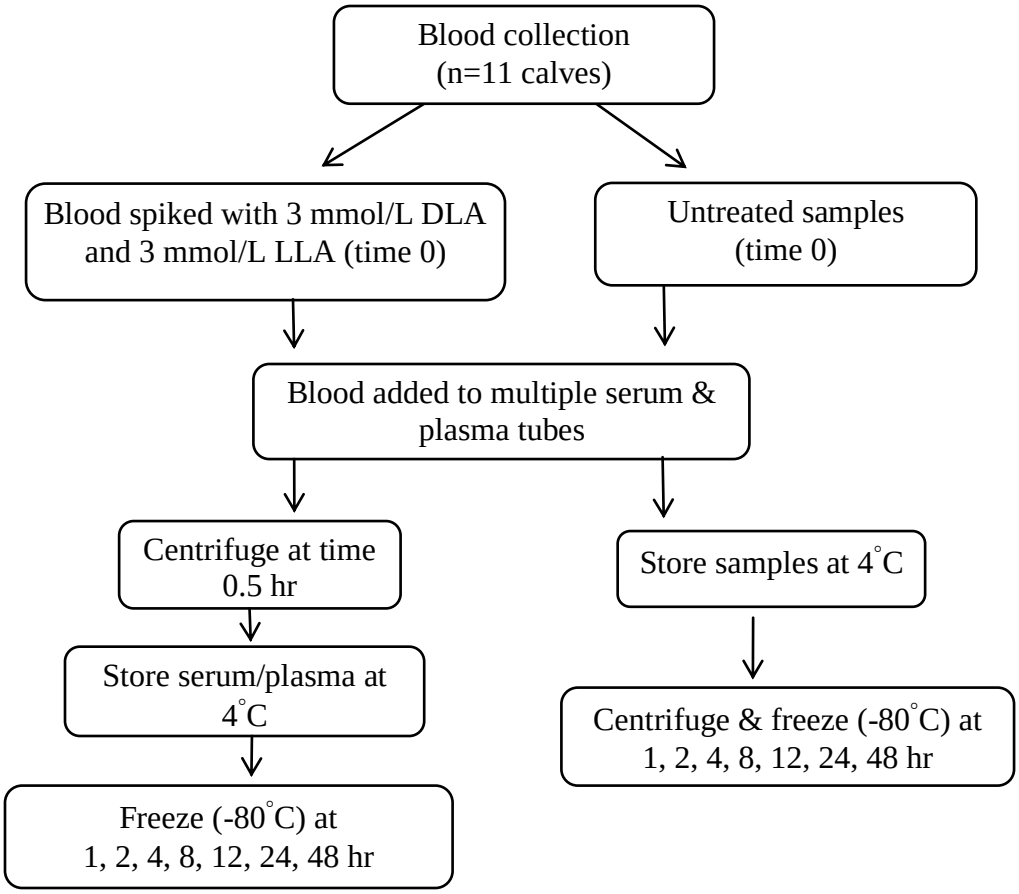

Figure 3.1 Schematic flow chart of the study design and sampling handling processes. DLA, D-lactate; LLA, L-lactate.

For separation of plasma or serum prior to storage, tubes were centrifuged at 2,000 x *g* for 15 minutes (Eppendorf 5804R Centrifuge; Eppendorf, Hamburg, Germany). Following centrifugation, 0.5 ml aliquots of plasma or serum (from now on referred to as "separated" samples), respectively, were placed into 1.5mL microcentrifuge tubes (Eppendorf; Hamburg, Germany) and then stored at 4°C in a refrigerator alongside the uncentrifuged samples (from now on referred to as plasma or serum stored "in contact with cells"). One aliquot from each processing method was removed from the refrigerator and frozen at -80°C at times 1, 2, 4, 8, 12, 24 and 48 hours after collection. Prior to freezing, samples stored in contact with blood cells were centrifuged at 2,000 x *g* for 15 minutes (AccuSpin Micro 17; Fishers Scientifica, Schwete,

Germany) and plasma and serum harvested. As the initial blood processing time following collection varied, 15 minutes to 1 hour, for statistical comparison, the 1-hour sample was used as a baseline concentration.

After thawing, D- and L-lactate concentration in serum and plasma samples was measured using enzymatic assay kits as per manufacturer instructions (Biovision, Mountain View, CA, USA). The lower and upper detection limits of the assay kits are 0.01-10 mmol/L and 0.02-10 mmol/L for D-lactate and L-lactate respectively. In order to obtain accurate measurements within the assay kit detection limits, the serum and plasma spiked with DL-lactic acid were diluted accordingly. The dilution factors in the spiked serum and plasma samples were taken into account in the final calculations.

3.2.3 Enzymatic Kit Validation

The enzymatic assays for D- and L-lactate were validated by two researchers. Within each assay run, lactate standards and blank sample were included for the calibration curve. Positive and negative controls, standards, blanks, samples, and sample background were prepared as per manufacturer's instructions and analyzed in duplicate for each run. Standards were prepared by diluting an aqueous D-lactate solution (10 mmol/L) with assay buffer in concentrations of 500 µmol/L, 250 µmol/L, 100 µmol/L, 50 µmol/L, 25 µmol/L, and 10 µmol/L as per manufacturer's recommendations. A calibration curve was made in duplicate with each run and D- and L-lactate concentrations were determined from the calibration curve. Within-run and between run precision was assayed in each batch by using positive controls in duplicate (CV was less than 15% for both D- and L-lactate respectively). The mean recovery for the D-lactate assay ranged from 96%-101% and for the L-lactate assay it ranged from 99.1%-104.8%.

3.2.4 Statistical Analysis

Results are presented as mean ± standard deviation (SD) for all animals. The time-dependent changes of total, D-lactate, and L-lactate concentrations in samples were analyzed using an Analysis of Variance (ANOVA) with Ryan-Einot-Gabriel-Welsch F-test as the *post hoc* test. As there was variability in blood processing time following initial collection, the 1 hour time point was chosen as the baseline concentration value to which all subsequent concentrations were compared. A *P*-value of < 0.05 was considered to be statistically significant. The changes

in total lactate concentrations over time were further analyzed by linear regression. As both serum and plasma samples from a subset of calves (n=5) were obtained at the same time during collection, the differences between D- and L-lactate concentrations in serum and plasma were analyzed by a paired t-test. Again, a *P*-value of < 0.05 was considered to be statistically significant. All statistics were calculated, and graphs created, using GraphPad Prism 5 for Windows (GraphPad Software, San Diego California, USA) and SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

3.3 Results

When comparing pooled D-lactate concentrations at all time points between untreated serum and plasma samples stored under the same conditions, D-lactate concentration in serum samples stored in contact with blood cells (Figure 3.2[A]) and in separated serum samples (Figure 3.2[B]) were found to be 13.67 % and 13.51% higher, respectively, than corresponding plasma D-lactate concentrations ($P < 0.05$). In contrast, L-lactate concentrations in untreated plasma samples stored in contact with blood cells were found to be 11.78% higher than corresponding serum L-lactate concentration ($P < 0.05$; Figure 3.3[A]) There was no difference between serum and plasma L-lactate concentrations in untreated samples stored separated from blood cells (Figure 3.3[B]).

In untreated calf serum samples stored in contact with blood cells, total lactate concentrations (calculated by addition of measured D- and L-lactate concentrations) increased significantly by 76.4% (4.45 mmol/L compared to 2.52 mmol/L) at 48 hours when compared to 1 hour (Figure 3.4 [A]). No significant differences were observed in total lactate concentrations when blood was centrifuged following collection and serum was stored separated from blood cells (Figure 3.4[A]). While a similar trend was observed in plasma samples, the increase over time in total lactate concentration in samples stored in contact with blood cells was not significant (Figure 3.4 [B]).

D- and L-lactate concentrations in all samples stored separated from blood cells were stable up to 48 hours when stored at 4°C (Figures 3.5, 3.6, 3.7 & 3.8). D-lactate in untreated serum samples stored in contact with blood cells was significantly increased at 48 h (increase of 82.3%; 0.708 mmol/L compared to 0.377 mmol/L; $P < 0.05$) compared to the 1 hour samples. (Figures 3.5 [A]). Although there appears to be an increasing trend in D-lactate concentration in

untreated plasma samples stored in contact with cells (0.535 mmol/L compared to 0.315 mmol/L), this increase was not found to be significant (Figure 3.5[B]).

In untreated serum samples left in contact with blood cells, L-Lactate concentrations increased significantly by 74.4% (3.75 mmol/L compared to 2.15 mmol/L) but not in separated samples (Figure 3.7[A]). In untreated plasma samples left in contact with blood cells, a similar trend was observed but the increase in L-lactate was not found to be significant (Figure 3.7[B]). In spiked samples stored in contact with blood cells, a significant increase in L-lactate concentration at 48 h in samples stored in contact with blood cells was observed in both serum (39.4% increase; 7.26 mmol/L compared to 5.21 mmol/L; $P < 0.05$; Figure 3.8[A]) and plasma (40.2% increase; 7.01 mmol/L compared to 5.00 mmol/L; $P < 0.05$; Figure 3.8[B]) samples.

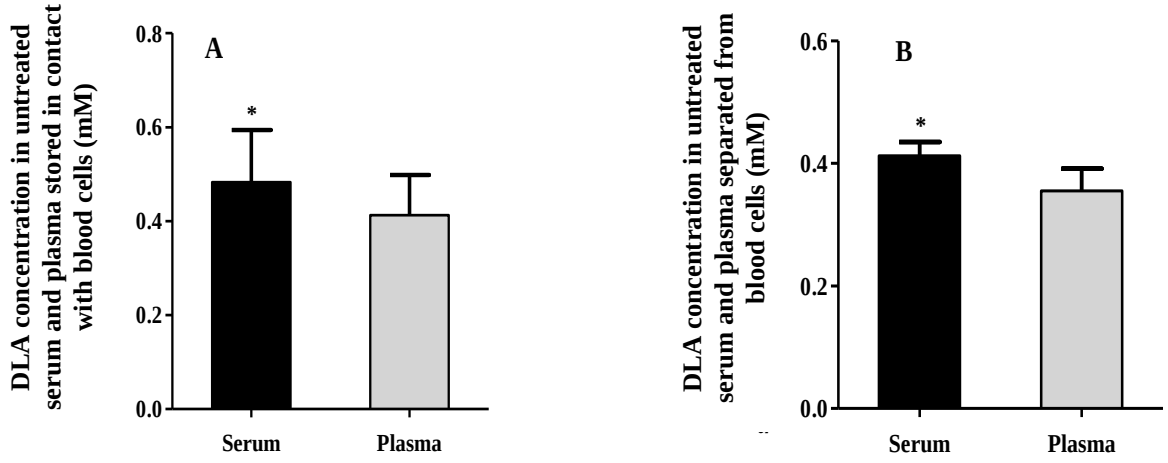

Figure 3.2 D-Lactate (DLA) concentration (mean ± SD, n=5) in untreated calf serum and plasma samples stored in contact with (A) or separated from (B) from blood cells. Each bar in the graph represents the mean D-lactate concentrations at all time points in the specific sample set. *Significant at P <0.05 when compared to plasma using a paired t-test.

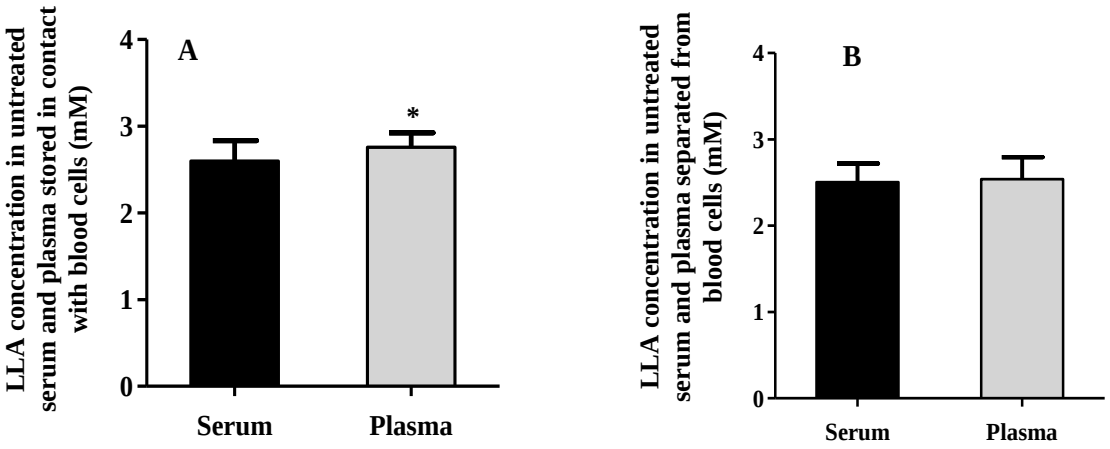

Figure 3.3 L-Lactate (LLA) concentration (mean ± SD, n=5) in untreated calf serum and plasma samples stored in contact with (A) or separated from (B) from blood cells. Each bar in the graph represents the mean D-lactate concentrations at all time points in the specific sample set. *Significant at P <0.05 when compared to plasma using a paired t-test.

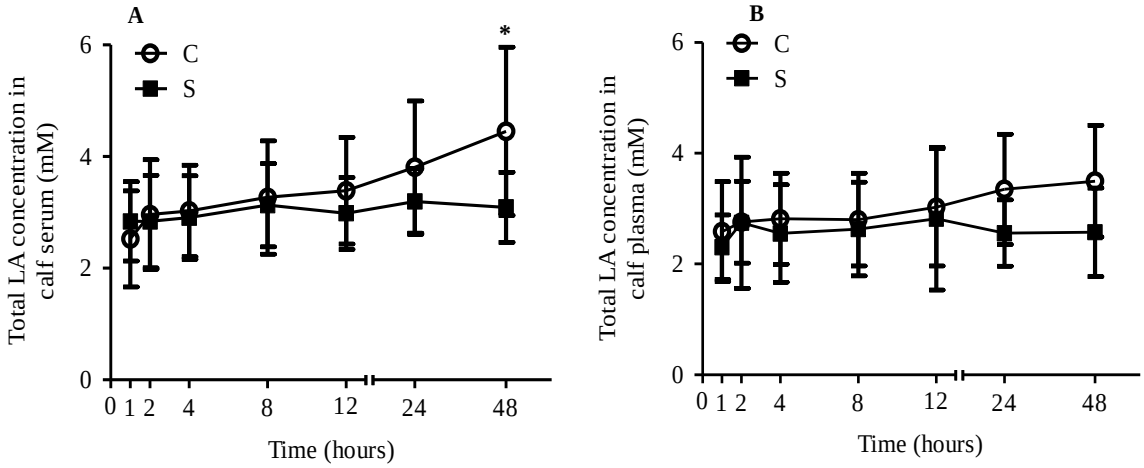

Figure 3.4 Total lactate (LA) concentration (mean ± SD, n=8) in untreated calf serum (A) and plasma (B) stored in contact with (contact, C) or separated from (separated, S) blood cells at 1, 2, 4, 8, 12, 24, and 48 hours. *Significant at P <0.05 using Ryan-Einot-Gabriel-Welsch multiple F test. Total lactate (LA) was calculated by adding [D-lactate] and [L-lactate].

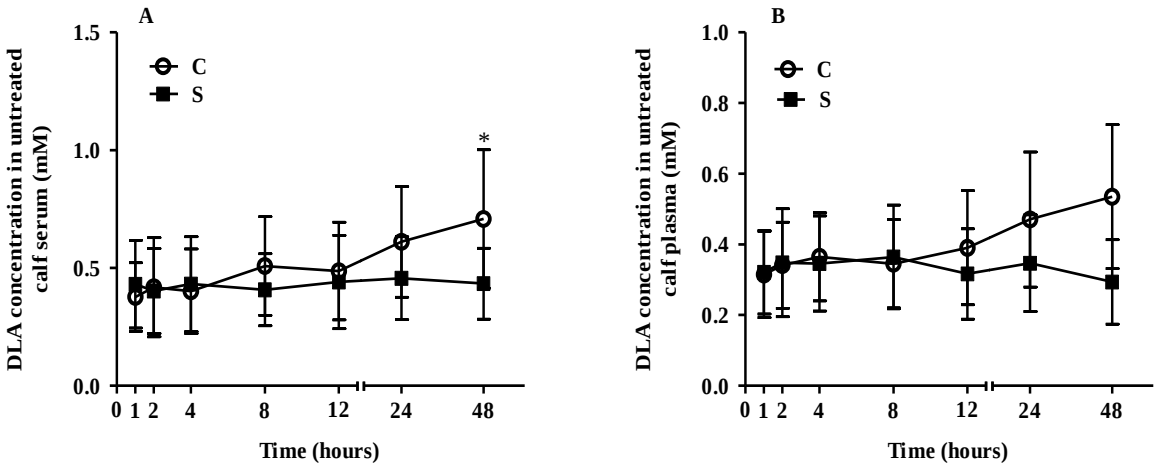

Figure 3.5 D-Lactate (DLA) concentration (mean ± SD, n=8) in untreated calf serum (A) and plasma (B) samples stored in contact with (contact, C) or separated from (separated, S) blood cells at 1, 2, 4, 8, 12, 24, and 48 hours. *Significant at P <0.05 using Ryan-Einot-Gabriel-Welsch multiple F test.

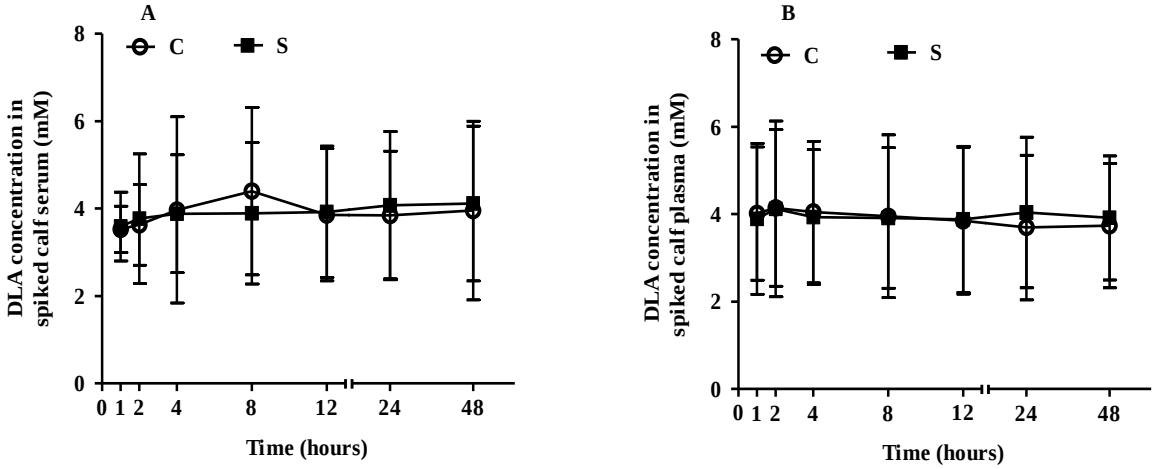

Figure 3.6 D-Lactate (DLA) concentration (mean ± SD, n=8) in spiked calf serum (A) and plasma (B) samples stored in contact with (contact, C) or separated from (separated, S) blood cells at 1, 2, 4, 8, 12, 24, and 48 hours.

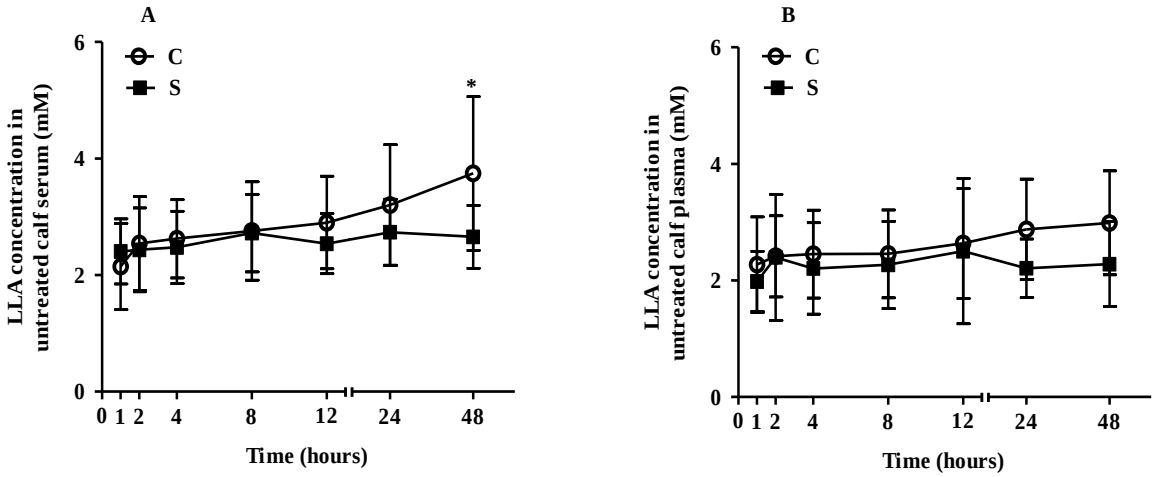

Figure 3.7 L-Lactate (LLA) concentration (mean ± SD, n=8) in untreated calf serum (A) and plasma (B) samples stored in contact with (contact, C) or separated from (separated, S) blood cells at 1, 2, 4, 8, 12, 24, and 48 hours. *Significant at P <0.05 using Ryan-Einot-Gabriel-Welsch multiple F test.

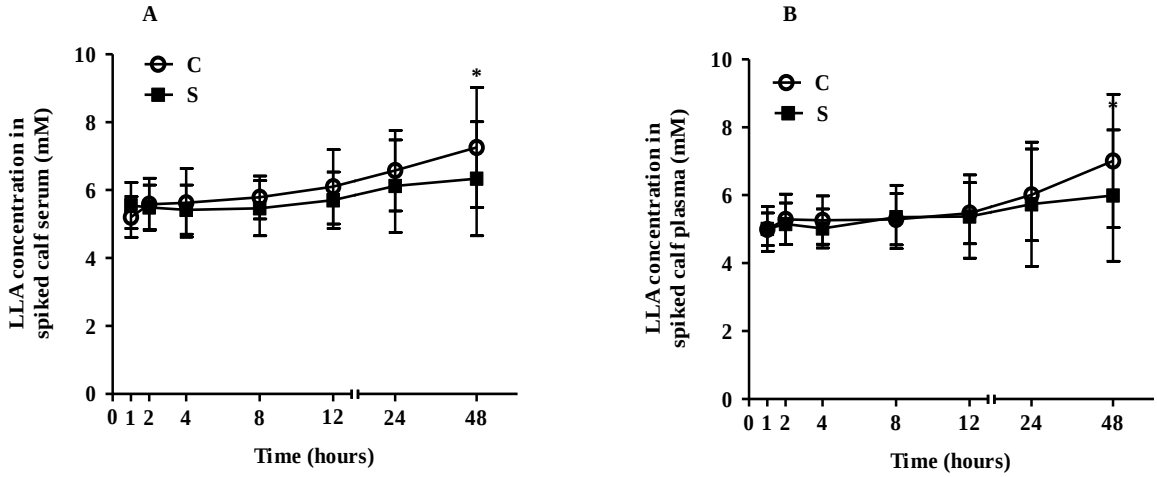

Figure 3.8 L-Lactate (LLA) concentration (mean ± SD, n=8) in spiked calf serum (A) and plasma (B) samples stored in contact with (contact, C) or separated from (separated, S) blood cells at 1, 2, 4, 8, 12, 24, and 48 hours. *Significant at P <0.05 using Ryan-Einot-Gabriel-Welsch multiple F test.

3.4 Discussion

For some assays, serum and plasma samples are considered to be equivalent while for others (i.e. bile acids, potassium, aldolase, lactate dehydrogenase) results obtained from serum and plasma samples can differ significantly to the point that clinical decisions are altered (Miles, Roberts, Putnam & Roberts, 2004). In this study, D-lactate concentration in calf serum left in contact with, and separated from, blood cells were 13.67 % and 13.51% higher respectively when compared to plasma. L-lactate concentration in calf plasma stored in contact with blood cells was 11.78 % higher when compared to calf serum stored in contact with blood cells. The magnitude of change between plasma and serum D- and L-lactate concentrations is not likely large enough to alter clinical decisions and treatment. However, although the difference between plasma and serum in D- and L-lactate concentrations may be small, it is important to establish a standard collection method and samples should be handled in the same way throughout the entire analytical process (Tuck et al., 2009).

In laboratory investigations, stability has been defined as "the capability of a sample for analysis to retain the initial property of a measured constituent for a period of time within specified limits when the sample is stored under defined conditions" (WHO, 2002). Processing and storage of biological samples can have significant effects on stability and analytical reliability. Identification of pre-analytical conditions that cause significant increases in D- and L-lactate concentrations can be useful in preventing unreliable research conclusions, assessments of D- and L-lactate concentrations, and potentially prevent unnecessary treatment interventions. This study was conducted for two reasons. First, elevated concentrations of D-lactate, rather than L-lactate, are associated with the neurological dysfunctions observed in diarrheic calves with acidosis and secondly, although samples are often collected outside of a clinical/laboratory setting, little is known about the effect of pre-analytical processing and storage on the stability specific D- and L-lactate isomers. This study investigated the stability of both lactate isomers, D- and L-lactate, over time, in serum and plasma samples stored at 4°C. Samples were stored after separation by centrifugation or in contact with blood cells.

In this study, total lactate increased over time in serum samples stored in contact with blood cells which is consistent with the literature. The concentration difference reached statistical significance ($p < 0.05$) at 48 hours when compared to the 1 hour reference samples. At room temperature, Boyanton and Blick (2002) reported that serum or plasma with prolonged

contact with blood cells prior to centrifugation and analysis can result in significant increases in total lactate concentrations at four hours. The difference in time reaching statistical significance may be due to the difference in study storage temperatures, as colder temperatures have been shown to slow the production of lactate (Astles et al., 1994), and may suggest a species and age difference.

This study further demonstrated that D-lactate concentrations in untreated serum samples stored in contact with blood cells increased over time and differed significantly from the 1 hour reference samples at 48 hours ($P < 0.05$). Though not statistically significant, a trend towards increased D-lactate concentration over time was also seen in the untreated plasma samples stored in contact with blood cells. D-lactate production may continue after blood is collected and stored as a result of the glyoxylase metabolic pathway. This pathway is present in the cytosol of all cells, including erythrocytes (McLellan & Thornalley, 1989; Thornalley, 1993). The glyoxylase system detoxifies and catalyses the conversion of methylglyoxal, a reactive glycating agent, formed when carbohydrates, lipids, and amino acids are metabolized. Through this system, methylglyoxal is metabolized to D-lactate (Kalapos, 2008; Xue et al., 2011; McLellan & Thornalley, 1989). Increases in sample concentration of D-lactate due to this ongoing glyoxylase system in cells will likely be progressive over time since, once accumulated *in vitro*, D-lactate cannot undergo further metabolism (Karg et al., 2009; McLellan & Thornalley, 1989). One can speculate that continued storage of plasma samples would have eventually resulted in a significant rise in D-lactate concentrations.

This study also observed that L-lactate concentration increased over time, in untreated and spiked serum samples, and in spiked plasma samples stored in contact with blood cells. This progressive increase in blood L-lactate is likely due to ongoing glycolysis, *in vitro*, between the time of blood collection and measurement of lactate concentration. After the collection of blood, if plasma and serum are left in prolonged contact with cells, additional L-lactate can be produced from glucose by all cellular constituents of blood, including platelets, through ongoing glycolysis (Astels et al., 1994). Glycolysis is the metabolic pathway by which glucose is converted to pyruvate and the enzymes required for this process are found in the cytoplasmic compartment of cells. Pyruvate in collected blood will be reduced to lactate by the enzyme lactate dehydrogenase. In this process, NADH is oxidized to NAD^+ and becomes available for the glyceraldehyde- 3-phosphate dehydrogenase reaction in the glycolysis pathway. As a result,

blood glucose concentration will decrease and L-lactate concentration will increase over time (Horton et al., 2002; Astels et al., 1994). However, as this study did not measure glucose concentrations, we can only speculate that this may be one of the mechanism by which this study observed, over time, increases in L-lactate concentration. In addition, although spiked blood samples were examined in this study and showed a significant difference in serum and plasma L-lactate concentration at 48 hours, care should be taken when extrapolating these results to subjects with clinical cases of acidosis. Further studies should investigate the storage dependent changes in D- and L-lactate concentrations in serum and plasma, over time, in animals with clinical acidosis.

As many blood components (i.e. proteins, enzymes, hormones, biochemical substance) are generally more stable at low temperatures and in serum/plasma, it is recommended that following collection, cells be separated from serum or plasma and kept cool or frozen during transport to the laboratory for analysis (Peng et al., 2010; Stahl & Branslund, 2005). This study demonstrated that both D- and L-lactate concentrations in calf serum and plasma following separation from blood cells 0.5 hours following collection can be measured for up to 48 hours when stored at 4°C. The stability of both D- and L-lactate concentrations may be explained by the immediate separation of plasma and serum from cells which would likely prevent ongoing production of L-lactate via glycolysis and/or D-lactate via glyoxylase pathway in the cytoplasm of cells following collection as previously described. Other studies have demonstrated total lactate concentrations to be stable in human serum and plasma samples immediately separated from cells for up to 56 hours at room temperature (25°C) (Boyanton & Blick, 2002) and in rat plasma samples at 4°C (Peng et al., 2010) however this study's observations on the stability of specific lactate enantiomers, D- and L-, following centrifugation and storage at 4°C is new.

In some areas, access to a centrifuge for pre-analytical processing may not be available (i.e. developing countries, field work) and past research has recommended the use of heparinized whole blood for lactate analysis (WHO, 2002; Wiese et al., 1997). This would eliminate the need for centrifugation, however, colorimetric enzymatic assay kits, like the ones used in this study, could not be used to determine lactate concentrations In these readily available lactate kits, D- and L-lactate is oxidized by L-lactate dehydrogenase and D-lactate dehydrogenase respectively and generates proportional color. An alternate analysis of lactate could be used for whole blood (i.e. blood gas analyzers, portable lactate analyzers); however, these analytical

procedures, like centrifuges, may not be available and do not distinguish between the D- and L-lactate isomers. Even in whole blood, it is well known that delays in processing anywhere from as little as fifteen minutes to one hour can result in significant increases in total lactate concentrations (Calatayud & Tenís, 2003; Toffaletti et al., 1992; Astles et al., 1994).

As this study investigated storage at 4°C, further research could investigate the use of different storage temperatures as some remote areas may not have access to refrigeration immediately following collection (i.e. ice packs, crushed ice). The use of ice packs and wet ice have shown to slow down, but not eliminate, the production of total lactate however, again, the stability of specific D- and L- lactate isomers at these variable temperatures remains unknown (Seymour et al., 2011, Astles et al., 1994; Andersen et al., 2003; Calatayud & Tenís, 2003). Future research could also explore the use of different anticoagulants and/or antiglycolytic agents in test tubes for analysis of D- and L-lactate concentrations. Astles et al. (1994) demonstrated that the use of antiglycolytic agents, sodium fluoride and potassium oxylate, maintained stable lactate concentrations at room temperature for up to eight hours while le Roux et al. (2004) demonstrated that collection tubes treated with sodium fluoride, glyceraldehydes, and potassium oxylate could inhibit glycolysis for up to 24 hours at room temperature prior to centrifugation. While the use of different antiglycolytic and anticoagulant agents could be explored for use in research, these tubes are not commonly available and may not be feasible and/or available for use in remote areas or in a clinical/diagnostic setting.

It is important to have standard blood collection and processing procedures to ensure reliable and accurate results for proper clinical diagnosis and/or treatment in the clinical setting and for research. This study was the first to investigate the stability of individual lactate isomers, D- and L-, over time, stored with or without contact with blood cells. Given the results of this study, to ensure a reliable measurement of D- and L- lactate concentrations, serum and/or plasma samples should be centrifuged and separated from blood cells as soon as possible following collection to stabilize the samples, but can thereafter be stored at 4°C for up to 48 hours without noticeable changes in D- or L-lactate concentrations.

CHAPTER FOUR
D-LACTIC ACIDOSIS IN PEDIATRIC DIARRHEA

4.1 Introduction

Acute diarrhea remains a public health concern as it continues to be a major cause of mortality and morbidity among children worldwide. Although the mortality rate of children under five years of age has decreased, diarrhea continues to be the second leading cause of child death due to infectious disease following pneumonia (Black et al., 2010; UNICEF & WHO, 2004). According to Black et al. (2010), diarrhea accounts for an estimated 1.336 million deaths globally, approximately 15% of total child deaths, with 51% of deaths occurring in India, Nigeria, Afghanistan, Pakistan, and Ethiopia. Pediatric diarrhea has many causes; however, the majority of reported cases are due to infections of the gastrointestinal tract and are attributed to viral agents (Guandalini, 2005; Malek et al., 2006). Despite the cause, diarrhea can result in clinical consequences including electrolyte disturbances, dehydration, and metabolic acidosis and, if left untreated, can lead to death.

Metabolic acidosis as a result of diarrhea was initially thought to be caused by an overproduction of L-lactate in the tissues by anaerobic metabolism due to dehydration and/or loss of bicarbonate in the feces (Kasari, 1999; Perez et al., 1987). Research in neonatal animals has shown that metabolic acidosis in diarrhea can occur in the absence of dehydration with associated high blood levels of D-lactate (Kasari & Naylor, 1984; Kasari & Naylor, 1986; Omole et al., 2001; Ewaschuk et al., 2003; Ewaschuk et al., 2004a). D-Lactate is produced primarily by microorganisms in the gastrointestinal tract, with very small amounts produced endogenously via the methylglyoxal pathway (Uribarri et al., 1998; Thornalley, 1993). Elevated concentrations of fecal D-lactate provide evidence that gastrointestinal bacterial fermentation contributes to the development of acidosis found in diarrheic animals (Omole et al., 2001; Ewaschuk et al., 2004a).

D-Lactic acidosis in humans has been defined as metabolic acidosis that accompanies an increase in a serum D-lactate concentration greater than 3 mmol/L (Uribarri et al., 1998). It is a rare clinical condition that is most often reported as a clinical complication of short bowel syndrome (SBS). Grünert et al. (2010) recently reported a case of D-lactic acidosis with acute encephalopathy in a child with a carbohydrate malabsorption syndrome of an unknown cause. Signs and symptoms of D-lactic acidosis appear to be non-specific and can vary in duration.

Symptoms reported include slurred speech; blurred vision; confusion; disorientation; delirium; dizziness; ataxia; lethargy; lack of concentration; somnolence; hallucinations; weakness; unsteady gait; irritability; and abusive/hostile behaviour (Uribarri et al., 1998; Hove & Mortensen, 1995; Stolberg et al., 1982; Al Chekakie et al., 2004). D-lactic acidosis has also been associated with episodes of encephalopathy in humans (Htyte et al., 2011; Grünert et al., 2010; Munakata et al., 2009; Uribarri et al., 1998) and, in animal studies; high infusions of D-lactate can cause acute neurocardiac toxicity (Abeysekara et al., 2007).

Identifying D-lactic acidosis in children with acute diarrhea in the absence of SBS has been poorly examined. This warrants investigation because, like SBS and congenital carbohydrate malabsorption disorders, infectious diarrhea (i.e. Rotavirus) may result in carbohydrate malabsorption and acidosis with little detectable fecal bicarbonate (Sack et al., 1982). The objective of this study was to determine whether D-lactic acidosis occurs in children with acute diarrhea and metabolic acidosis. Blood obtained from children was analyzed for the blood parameters that define metabolic acidosis and included concentrations of D-lactate, L-lactate, and pyruvate. Fecal D-lactate concentrations were also to be determined. To investigate fecal D-lactate concentrations from samples collected in disposable diapers containing polyacrylate granules (gel) a small study was conducted as there is no known method available to exact D-, L-lactate and pyruvate from disposable diapers. The objective of this was to determine the recovery of D-lactate from a disposable diaper. If D-lactic acidosis occurs in children with acute diarrhea, treatment strategies could be altered, and/or compliment current treatments (i.e. oral and/or parenteral rehydration), to target D-lactate producing bacteria and reduce the clinical complications of D-lactic acidosis.

4.2 Materials and Methods

4.2.1 Study Design

All infants and/or children (≤ 5 years of age) with acute diarrhea, as defined by the World Health Organization (2009) as “three or more loose liquid stools per day or more frequently than normal”, and suspected metabolic acidosis were eligible to participate in the study. The sample size goal was 30 subjects in order to provide data with statistical significance as determined by previous D-Lactate studies conducted at University of Saskatchewan (Omole et al., 2001; Ewaschuk et al., 2003; Ewaschuk et al., 2004a; Abeysekara, 2009). Study participants were to

be recruited from Royal University Hospital (RUH) in Saskatoon, Saskatchewan. To minimize invasiveness, diarrhea was to be severe enough to warrant routine clinical investigation of blood electrolytes. This blood was then to be saved for analysis of lactate isomers and pyruvate without further subject burden. To obtain a wide range of diarrhea severity, much work was completed to initiate a similar study to that of RUH in Awassa, Ethiopia as more severe and prolonged diarrhea is likely to occur in children living in developing countries. Actions taken and progress on this study are found in Appendix B. This study was approved by the Biomedical Research Ethics Board at the University of Saskatchewan (Bio-REB # 03-932) and operational approval granted from the Saskatoon Health Region (Appendix A).

4.2.2 Study Participant Recruitment

Children (≤ 5 years of age) with acute diarrhea and suspected metabolic acidosis were recruited for the study by physicians, medical residents, and/or nursing staff in the pediatric emergency room (PedsER) and two pediatric acute care wards at RUH, Saskatoon, Saskatchewan. Study participants were initially recruited only through the PedsER however, to identify those children who may have been missed by the PedsER staff, recruitment expanded to include the two pediatric acute clinical care wards in November, 2010 (Appendix A). Once identified as being eligible for the study, the attending physician and/or the nutrition study coordinator described, in detail, the study to the child's parent(s) and/or legal guardian(s) and, if agreeable to participate, consent forms were reviewed and signed.

4.2.3 Data Collection

4.2.3.1 Medical Health Records

The medical health record of all study participants were reviewed for biochemical data associated with defining metabolic acidosis: sodium, potassium, chloride, bicarbonate (total carbon dioxide), and anion gap. These parameters were measured by RUH clinical biochemistry laboratory (Cobas 6000®; Roche Diagnostics, IN, USA) as per hospital protocol. As SBS is one of the few medical conditions in which D-lactic acidosis is a clinical complication, the medical condition and/or disease that the child may have may be useful information for future research. Medication history was also reviewed as diarrhea is associated with the use of some medications (i.e. antibiotics).

4.2.3.2 Assessment of Diarrhea Severity

This study adopted the use of the Bristol Stool Form Scale to assess diarrhea severity (Table 4.1). The scale classifies stool based on its consistency with seven categories ranging from "separate hard lumps like nuts" (Type 1) to "watery, no solid pieces, entirely liquid" (Type 7). Once consent was obtained, the participant's parent(s) and/or legal guardian(s) were asked to rate the child's feces using the Bristol Stool Form Scale.

Table 4.1 Description of Bristol Stool Rating Scale

Type 1	Separate hard lumps, like nuts (hard to pass)
Type 2	Sausage-shaped but lumpy
Type 3	Like a sausage but with cracks on its surface
Type 4	Like a sausage or snake, smooth and soft
Type 5	Soft blobs with clear-cut edges (passed easily)
Type 6	Fluffy pieces with ragged edges, a mush stool
Type 7	Water, no solid pieces. Entirely Liquid

Adapted from Lewis & Heaton, 1997.

4.2.4 Sample Collection and Analysis

4.2.4.1 Blood Samples

Once consent was obtained from the participant's parent(s) and/or legal guardian(s), the RUH biochemistry laboratory personnel were instructed to store the remaining pediatric blood samples, approximately 1mL, drawn for clinical electrolyte/blood gas measurements at -80°C (Appendix C). The reserved blood was drawn prior to initiation of any medical treatment (i.e. oral and/or parental rehydration solution). All samples were transported to the Irwin Nutrition Laboratory at College of Pharmacy and Nutrition, University of Saskatchewan and stored at -80˚C until analysis. After thawing, samples were analyzed for D-lactate, L-lactate, and pyruvate using commercially available enzymatic assay kits following the manufacturer instructions (Biovision, Mountain View, CA, USA). The detection limits of the D- lactate, L-lactate, and

pyruvate assay kits are 0.01-10 mmol/L; 0.02-10 mmol/L; and 1-200 µmol/L, respectively. When available, blood gas and electrolyte measurements (i.e. Na^+, K^+, Cl^-, CO_2, AG) of the reserved blood sample was obtained from the study participants' medical health record.

4.2.4.2 Fecal Samples

Fecal samples were obtained by collecting the study participants' disposable diaper containing diarrheic feces. This simple stool collection method was chosen to encourage recruitment of study participants and reduce hospital staff burden. Diapers were temporarily stored on the hospital ward at -20°C and transported to the Irwin Nutrition Laboratory at College of Pharmacy and Nutrition, University of Saskatchewan and stored at -80˚C. As there is no known method available to extract the organic acids, D-lactate, L-lactate and pyruvate, from disposable diapers a small experiment was conducted to determine whether or not the compounds of interest could be measured.

A clean, dry diaper was cut into two, 3 cm x 3 cm test strips. The two test strips had three distinct layers including a topsheet, acquisition/distribution layer containing polyacrylate granules (gel), and backsheet. One test strip was spiked with 10 mL of 10 mmol/L D-lactate. D-Lactate was allowed to soak into the test strip for approximately ten minutes. The diaper liner (topsheet) was removed from the test strip and centrifuged in a 50 mL centrifuge tube (Falcon™; BD) at 14,000 rpm for 20 minutes. Liquid extracted from the liner (12.5 µl) was then analyzed for D-lactate using an enzymatic assay kit as per manufacturer instructions (Biovision, Mountain View, CA, USA). One gram of diaper polyacrylate granules (absorbent gel) from the core of the diaper was weighed from the remaining test strips and spiked with 10 mL of 10 mmol/L of D-lactate. D-Lactate was allowed to soak into the gel for approximately ten minutes. The gel was then dissolved in 20 mL of distilled water and centrifuged in a 50 mL centrifuge tube (Falcon™; BD) at 14,000 rpm for 20 minutes. Liquid extracted from the diaper gel (12.5 µl) was analyzed for D-lactate using an enzymatic assay kit (Biovision, Mountain View, CA, USA).

In this small experiment, 36.5% and 91.2% of the D-lactate spiked to the disposable diaper liner and polyacrylate granules (gel) in test strip #1 and test strip #2, respectively, was recovered (Table 4.2). Although the compound of interest, D-lactate, could be recovered from the disposable diaper, it is unknown if the study participants' diapers collected contained urine. D-lactate is known to be excreted in the urine of healthy infants and children with excretion

highest during the first year of life (Henry et al., 2011; Haschke-Becher et al., 2000; Haschke-Becher et al., 2008). Therefore, if the study participants' diapers were analyzed for D-lactate, there may be an overestimation of D-lactate concentrations due to the presence of urine. Due to the homogeneity of fecal samples collected and the unknown presence of urine in the disposable diaper, study participants' fecal samples were not analyzed for D-, L-lactate or pyruvate.

Table 4.2 Fecal D-lactate concentrations from two disposable diaper test strips spiked with 10 mmol/L D-lactate

	D-lactate (mmol/L)	% Recovery
Test Strip #1: Diaper Liner (top sheet)	0.0365	36.5
Test Strip #2: Diaper Gel in 20 mL water	0.0912	91.2

4.3 Statistical Analysis

Mean and standard deviation (SD) of all measured blood parameters were calculated using Microsoft Excel (Microsoft Corporation Inc., 2007).

4.4 Results

Between June 2009 and April 2011, nine study participants were recruited by physicians, medical residents and nursing staff at RUH. Subject characteristics including age, gender, and diagnosis at time of sample collection are found in Table 4.3. Consent was obtained from all study participants' parent(s) and/or legal guardian(s). No children were taking any medications known to be associated with diarrhea upon admission to RUH (i.e. antibiotics). Feces for all children (n = 9) in this study were rated as Type 7, "Water, no solid pieces, entirely liquid". One child, subject 008, in this study had an elevated anion gap, > 16 mmol/L, which suggests metabolic acidosis (Table 4.2); however, it should be noted that this sample was identified by the RUH biochemistry laboratory as being slightly hemolyzed. All study participants had D-lactate values higher than those found in the literature for healthy children (0.03 mmol/L-0.05 mmol/L)

(Connolly et al., 2005). The mean D-lactate concentration was three times higher when compared to healthy children and 1.5 times higher when compared to healthy adults, < 0.1 mmol/L, (Ewaschuk et al., 2005; Hove & Mortensen, 1995; McLellan et al., 1992; de Vrese & Barth, 1991; Herrera et al., 2008) in the literature. The mean of all other blood measures, L-lactate, pyruvate, Na^+, K^+, Cl^-, CO_2, and AG, were within reference range. No clinical cases of D-lactic acidosis were observed.

Table 4.3 Age, gender, and diagnosis of children (n=9) with acute diarrhea at time of sample collection

Subject	Age (years)	Gender	Diagnosis
001	0.83	N/A	Gastroenteritis
003	1	Male	Gastroenteritis
004	2	Female	Gastroenteritis
005	1	Male	Gastroenteritis
007	3	Female	Gastroenteritis
008	1	Female	Gastroenteritis
009	N/A	Male	N/A
010	2	Male	Dehydration secondary to gastroenteritis
011	1	Male	N/A
Mean (n=8)	1.48		
SD	0.773		

N/A, not available.

Table 4.4 Serum D- and L-lactate, pyruvate, and electrolyte concentrations in children (n=9), ≤ 5 years of age, with acute diarrhea

	D-lactate mmol/L	L-lactate mmol/L	Pyruvate mmol/L	Na^+ mmol/L	K^+ mmol/L	Cl^- mmol/L	CO_2 mmol/L	AG mmol/L
Literature values of healthy children	0.03-0.05* < 0.1 (adult)**	< 2.2	0.03-0.08	135-146	3.5-5.1	100-110	18-27	8-16
001	0.183	1.95	0.044	N/A	N/A	N/A	N/A	N/A
003	N/A	N/A	N/A	137	4.1	104	19	14
004	0.147	1.45	0.051	135	4.2	108	18	9
005	0.228	1.46	0.064	136	4.6	107	18	11
007	0.087	1.27	0.046	151	4.3	131	14	6
008	0.198	2.24	N/A	140	6.4	101	15	24
009	0.085	1.48	0.028	N/A	N/A	N/A	N/A	N/A
010	0.125	1.77	0.045	136	3.6	109	18	8
011	0.181	1.68	0.089	N/A	N/A	N/A	N/A	N/A
Mean	0.154	1.66	0.052	139	4.53	110	17	12
SD	0.052	0.317	0.02	6.05	0.971	10.7	2	6.48
	n = 8	n = 8	n = 7	n = 6	n = 6	n = 6	n = 6	n = 6

N/A, not available; AG, anion gap; *Connolly, Abrahamsson & Bjöorkstén (2005); ** Ewaschuk et al. (2005); Hove & Mortensen (1995); McLellan et al. (1992) de Vrese & Barth, (1991) Herrera et al. (2008).

4.5 Discussion

This study found no clinical cases of D-lactic acidosis in children 10 months to 3 years of age with acute diarrhea as blood D-lactate concentrations of all study participants was below 3.0 mmol/L. These results were not surprising as upon review of blood electrolytes, most biochemistry values were within reference range with no indication of metabolic acidosis. One study participant did have an elevated anion gap which may be indicative of an accumulation of organic acids; however, since this sample was flagged as slightly hemolyzed, values may have been overestimated. Hemolysis is usually caused by improper specimen collection, processing and/or transport and results in the breakage of the red blood cell membrane. As a result of this breakage, hemoglobin and other intracellular components leak into the surrounding fluid and cause false elevations of some analytes (i.e. ALT, AST, LDH, magnesium, phosphorus and potassium) or dilution effects (i.e. glucose, sodium, and chloride) (Lippi, Salvagno, Montagnana, Brocco, & Guidi, 2006). For this study, induced changes in potassium as a result of hemolysis could have led to a miscalculation of the anion gap.

A limitation of this study is that the acute diarrhea of the study participants may not have been severe enough to result in the overproduction of D-lactate in the gastrointestinal tract to result in elevated blood D-lactate concentrations. In this study, attempts were made to measure diarrhea severity of the study participants. Initially, the Bristol Stool Form Scale (BSFS) was used however this tool was validated in adults as a measure of stool transit time rather than as a means of classifying stool form or assessing/ rating diarrhea severity. It was evident early that study participants would all classified as a "type 7: water, no solid pieces, entirely liquid"; therefore no useful information regarding diarrhea severity was obtained using the BSFS in isolation. In the literature, Johnston, Shamseer, da Costa, Tsuyuki, & Vohra (2010) identified a lack of consensus on the measurements of pediatric acute diarrhea and there does not appear to be a standard, validated tool used to assess and/or rate severity in acute pediatric diarrhea. Upon consultation with Dr. Johnston, permission for the use of an instrument developed by thirty experts in the field of diarrhea and gastroenteritis was granted. This tool, adopted in respect to diarrhea frequency and duration from Bliss, Larson, Burr & Savik (2001), is known as the Disease Activity Index Pediatric Gastroenteritis (iPAG). The iPAG was adapted for this study and consisted of an assessment/scoring of: stool consistency; diarrheal frequency and duration;

vomiting frequency and duration; and fever (Appendix E). Use of this tool confirmed that diarrhea severity amongst the study participants was, in fact, homogeneous.

Although no clinical cases of D-lactate acidosis were part of this study, 66.7% of study participants had a D-lactate concentration greater than 0.1 mmol/L which, when compared to the literature, is higher than that found in healthy adults (de Vrese et al., 1990; Hove et al., 1995; Ewaschuk et al., 2005). There is limited data on blood D-lactate concentrations found in healthy children. Connolly et al. (2005) investigated the levels of D-lactate in the blood in infants (n=10) six and twelve months of age and reported a range of 0.03 mmol/L-0.05 mmol/L (Mean: 0.040 ± 0.018 mmol/L). This study investigated blood D-lactate levels in 24 infants supplemented with *Lactobacillus reuteri* ATCC 55730 (n=14) or a placebo (n=10) from birth. Blood D-lactate concentrations were determined at six months and again at 12 months. This substudy is part of a much larger double blind, placebo controlled clinical trial in infants (n=232) investigating the effect of exposure to *Lactobacillus reuteri* on the development of allergy symptoms in the first year of a child's life.

D-Lactate can be effectively metabolized in humans by the liver and is also is found to be excreted in the urine of children (Connor, et al., 1983; Oh et al., 1985; de Vrese et al, 1990; de Vrese et al, 1991; Uribarri et al., 1998; Ewaschuk et al., 2005; Haschke-Becher et al., 2000; Haschke-Becher et al., 2008; Henry et al., 2011). D-lactate metabolism requires transport-mediated mechanisms. Transport of D-lactate into the blood is achieved via proton-linked monocarboxylate transporters (Halestrap & Price, 1999). Once in the blood, MCTs play a role in the transport of D-lactate into the liver in which it can be metabolized to pyruvate via hepatic mitochondrial putative D-lactate dehydrogenase (de Bari et al., 2002). These hepatic metabolic processes undergo developmental maturation. As the study participants were eight months to three years of age, the increase in D-lactate concentrations in this study population may be a result of immature transport mechanisms. The impact of disease (i.e. diarrhea) on the normal development and maturation rate of enzyme and transporters involved in D-lactate metabolism and excretion is also unknown. This may warrant further investigation.

Past research in neonatal animals with diarrhea has shown a positive linear correlation between fecal and serum D-lactate concentrations. High levels of blood D-lactate appear after high levels are produced in the gastrointestinal tract and there is evidence to suggest that a gastrointestinal threshold exists for the absorption of D-lactate into the blood in diarrheic

neonatal animals (Abeysekara, 2009; Zello, Abeysekara et al., 2009). Based on this evidence, in addition to determining blood D-lactate concentration, this study was initially designed to investigate fecal D-lactate concentrations to determine whether or not this finding holds true in children with acute diarrhea. As discussed previously in the section 4.2.4.2, fecal samples were collected in the study participants' disposable diaper in attempt to increase recruitment of study participants and reduce the PedsER staff burden. This method of fecal collection presented a challenge to the researchers as, upon review of the literature, there is no known method available to extract the organic acids, D-lactate, L-lactate and pyruvate, from disposable diapers. Past investigations using disposable diapers are generally analyzed for microbiological purposes and the microorganism of interest can be obtained by scraping the diaper surface with a sterile scraper (Liang & Redlinger, 2003). Modern disposable diapers contain polyacrylate granules, a superabsorbent polymer, which can absorb up to 300 times its weight in water (Kosemund et al., 2009; European Disposables and Nonwovens Association, 2008). D-Lactate, L-lactate, and pyruvate are water soluble; therefore, these organic acids may distribute into the absorbent gel found in disposable diapers and by merely scraping the surface of the diaper the analysis could underestimate the actual concentrations of D-lactate, L-lactate and pyruvate in the fecal samples. Results from the small experiment described in 4.2.4.2 demonstrated that D-lactate is distributed into, and can be extracted, from a disposable diaper containing polyacrylate granules. D-Lactate was not bonded to the polyacrylate granules but rather distributed in the water phase surrounding the granules. As D-lactate was recovered in both the disposable diaper liner and polyacrylate gel, all diaper layers should be included in analysis. Although the compound of interest can be recovered, due to the homogeneity of samples collected, the absence of metabolic acidosis, and the unknown presence of urine in the disposable diaper, this study did not analyze fecal samples for D-lactate, L-lactate, or pyruvate.

This study is one of few to investigate whether or not D-lactic acidosis occurs in children, eight months to three years of age, with acute diarrhea in the absence of short bowel syndrome. Ewaschuk (2004) conducted a similar study. However, blood D-lactate concentrations were not detectable and/or not quantifiable due to the limit of quantification, 1.0 mmol/L, of the high performance liquid chromatography assay used. It is possible that the incidence of D-lactic acidosis in children with acute diarrhea may not be to the extent seen in neonatal animals. Perhaps this may be explained by differing gastrointestinal physiology, and/or metabolism of D-

lactate, that exists between species or perhaps it may be due, in part, that children are often assessed and treated for illness much sooner than that of animals. Regardless, this research does warrant further investigation as only nine children were recruited for this study, in which all samples were homogenous in regards to the severity of diarrhea. Future investigation with more varying diarrhea severity, including more severe cases with acidosis, may reveal that D-lactic acidosis does, in fact, occur in children with acute diarrhea and treatment strategies could be altered to target the D-lactate producing bacteria. Future studies should also investigate fecal D-lactate concentrations as it is possible that a high D-lactate concentration in a disposable diaper, regardless if the diaper contained urine or not, may still prove to be a useful biomarker for D-lactic acidosis. Conversely, it may also be beneficial to explore alternate fecal sample collection methods to accurately determine fecal D-lactate concentrations in children less than five years of age with acute diarrhea. As clinical signs and symptoms of D-lactic acidosis are variable and non-specific, if a gastrointestinal threshold for the absorption of D-lactate into the blood exists in children with acute diarrhea determination of fecal D-lactate concentration may prove to be a useful biomarker. Future research could investigate potential treatments to predict, and ideally prevent D-lactic acidosis and its clinical consequences from occurring.

CHAPTER FIVE
INVESTIGATION OF A POTENTIAL FECAL D-LACTATE THRESHOLD IN NEONATAL CALVES WITH DIARRHEA

5.1 Introduction

In humans and animals, D-lactate, a physiological isomer of lactate, is produced mainly through the bacterial fermentation of carbohydrates (i.e. fibre and incompletely digested starch, lactose, and proteins) in the gastrointestinal tract with small amounts produced endogenously through the glyoxalase pathway in the liver (Ewaschuk et al., 2005; Duncan et al., 1996; Thornalley, 1994). Under healthy physiological conditions, D-lactate does not pose a threat to the body's acid-base balance. However, in neonatal animals with diarrhea, elevated blood D-lactate concentrations, > 3 mmol/L, have been reported and is a significant contributor to metabolic acidosis (Omole et al., 2001; Ewaschuk et al., 2004a). High concentrations of fecal D-lactate in diarrheic calves suggest the gastrointestinal tract is the source of D-lactate production in D-lactic acidosis (Ewaschuk et al., 2004a; Omole et al., 2001).

The most common cause of death in neonatal calf diarrhea is dehydration and metabolic acidosis (Smith, 2009). D-Lactic acidosis in neonatal calves with diarrhea has been associated with impaired neurological dysfunction including disturbance of the palpebral and menace reflexes; depression with weakness and ataxia; impaired posture and behaviour (Kasari & Naylor, 1984; Lorenz, 2004). In serious cases, D-lactic acidosis has been associated with episodes of encephalopathy and coma (Forsyth et al., 1991; Grünert et al., 2010; Htyte et al., 2011; Jorens et al., 2004). Abeysekara et al. (2007) also reported that D-lactate has direct neurotoxic effect in calves; however, the exact mechanism is not yet known.

Recent research in diarrheic lambs and calves suggest that high levels of blood D-lactate appear only after high levels of D-lactate are produced in the gastrointestinal tract. In other words, a potential gastrointestinal threshold may exist for the absorption of D-lactate into the blood of diarrheic neonatal animals (Abeysekara, 2009; Zello, Abeysekara et al., 2009).
In these studies D-lactate was measured using an HPLC system as described by Omole et al. (1999) and Ewaschuk et al. (2002). For statistical purposes and the biphasic (break-point) regression analysis, concentrations that were not detectable by HPLC in this study were assigned a value of 0 and concentrations below the limit of quantitation were assigned an arbitrary value.

As many serum values were below the limit of quantitation, the analysis in these studies implies a straight line at low serum and fecal concentrations and does not appear to increase until fecal D-lactate concentrations reached approximately 10 mmol/L. It is not known if the calculated threshold concentrations for absorption would be similar if D-lactate concentrations were plotted using quantified values as opposed to the assigned value. Further investigation is also warranted to include more diarrheic animals with a wide range of diarrhea severity. The objective of this study is to examine a wide range of diarrhea severity to determine if a fecal D-lactate threshold exists in the gastrointestinal tract at which D-lactate enters the systemic circulation in neonatal calves with diarrhea. Identifying elevated fecal D-lactate levels, early on, in clinical cases of diarrhea may prove to be a useful biomarker to predict, and ideally prevent, D-lactic acidosis.

5.2 Materials and Methods

5.2.1 Calves

Blood and fecal samples were obtained from 17 diarrheic claves, less than four weeks of age, from a research farm in Quebec, Canada. The calves were housed in individual pens and fed a commercial milk replacer (crude protein: 22%; protein of animal origin: 22%; fat: 17%; sodium: 0.65%; Calcium: 0.8%; Phosphorus: 66%; Vitamin A 40,000 UI/kg; Vitamin D3: 4000 UI/Kg; Vitamin E: 150 UI/Kg) twice daily and did not have access to water or hay. Depending the severity of the diarrhea, oral antibiotics (a mixture of neomycin, tetracycline and colistin) may have been added to the milk replacer. Calves were selected for this study on the basis of having spontaneously occurring diarrhea. All calves were assessed (Appendix F) and assigned a clinical assessment score based on physical appearance and behaviour; food and water intake; rectal temperature; and fecal characteristics (Table 5.1). To ensure a wide variety of diarrhea severity, additional blood and fecal samples were obtained from ten calves, less than four weeks of age, admitted for treatment of diarrhea to the Western College of Veterinary Medicine at University of Saskatchewan. Again, these calves were selected on the basis of having clinical signs of diarrhea and assigned a clinical assessment score. This study was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use (Appendix A).

Table 5.1 Description of Clinical Assessment Score

Physical Appearance and Behavior		**Score for this calf**
0	Normal, bright and alert	
1	Mildly lethargic	
2	Dull, nasal discharge, not interested in food	
3	Recumbent, cannot be roused	
Food and water intake		
0	Normal	
1	Decreased, does not finish meal	
2	Eats less than half of meal	
3	Doesn't eat at all	
Rectal temperature		
0	Normal (T = 38-39)	
1	Fever (39 < T < 40)	
2	Fever (T > 40)	
3	Hypothermia (T < 37.5)	
Character of Feces		
0	Normal	
1	Soft but do not soak through bedding	
2	Watery (soak through bedding)	
3	Watery and animal appears dehydrated (eyes sunken)	
Other		
0	No abnormal findings	
2	Coughing, colic, wobbly etc.	

5.2.2 Sample Collection and Analysis

5.2.2.1 Blood Samples

Blood samples were collected from each calf prior to any medical treatment. Blood was drawn (5-10 mL) from the jugular vein of each diarrheic calf and allowed to clot at room temperature for 20 minutes. Once clotted, the blood was centrifuged for fifteen minutes at 1500×*g*, serum transferred to a clean tube, and frozen at -80°C until analysis. Serum samples from Quebec, Canada were transported on dry ice to the Irwin Nutrition Laboratory, University of Saskatchewan. All samples were stored frozen at -80°C until analysis. After thawing, all blood samples were analyzed for D- and L-lactate using commercially available enzymatic assay kits following the manufacturer instructions (Biovision, Mountain View, CA, USA). The lower

and upper detection limits of the assay kits are 0.01-10 mmol/L and 0.02-10 mmol/L for D-lactate and L-lactate respectively.

5.2.2.2 Fecal Samples

Approximately 10-15 g of feces was collected from each diarrheic calf. In Quebec, immediately following collection, liquid nitrogen was used to snap-freeze each fecal sample. Samples were then transported to the Irwin Nutrition Laboratory, University of Saskatchewan on dry ice and stored at -80°C until analysis. Fecal samples (10-15 g) were also collected from diarrheic calves at the University of Saskatchewan and immediately frozen at -80°C until analysis. On the day of analysis, samples were thawed on ice and homogenized in water (weight to volume ratio of 1:4) using a Polytron homogenizer (Brinkmann Instruments; Rexdale, Canada) for one minute. The homogenate was then centrifuged at 2,000×*g* for ten minutes at room temperature (AccuSpin Micro 17; Fishers Scientifica, Schwerte, Germany). The supernatant was collected and filtered through a 10K VWR® centrifugal filter (VWR International, LLC; Radnor, PA, USA) at 2,000 × *g* for ten minutes. The filtrate was then collected and analyzed for D- and L-lactate using enzymatic assay kits as per manufacturer instructions (Biovision, Mountain View, CA, USA). In order to obtain accurate measurements within the D- and L-lactate assay kit detection limits, the filtrate was diluted with D- or L-lactate assay buffer supplied by the manufacturer.

5.3 Statistical Analysis

D- and L-lactate concentrations of blood and fecal samples are presented as mean ± standard deviation (SD) and were calculated using Microsoft Excel (Microsoft Corporation Inc., 2007). To quantify the relationship between serum and fecal D- and L-lactate concentrations and between fecal D- and fecal L-lactate concentrations, Pearson's product moment coefficients of correlations were determined. A linear regression analyzed the relationships. All graphs were created, using GraphPad Prism 5 for Windows (GraphPad Software, San Diego California, USA).

5.4 Results

At the time of sample collection, calves (n=17) from Quebec appeared normal, bright and alert with normal food and water intake with the exception of one calf, whose physical appearance and behaviour was dull with nasal discharge; not interested in food and did not eat at all (Table 5.2). Rectal temperature was normal (38-39°C) for all calves. In regards to fecal characteristics, all calves were scored as 2: watery (soak through bedding) feces. Collectively, diarrheic calves had biochemical parameters within normal range (Table 5.4). Upon review of individual calves, two calves had a pH < 7.35, which is indicative of metabolic acidosis, but there were no clinical cases of D-lactic acidosis. The anion gap, which is often used to diagnosis metabolic acidosis, could not be calculated as chlorine was not determined during the blood gas analysis.

Calves (n=9) from the University of Saskatchewan exhibited a wide range of diarrhea severity, from mild to severe (Table 5.3). One calf did not have a clinical assessment. Calves had varying physical characteristics ranging from normal, bright, and alert to recumbent. Fecal characteristics also varied and scored from 0 to 3: 'normal' to 'watery' and 'animal appears dehydrated'. In regards to dehydration, calves were clinically assessed to be mildly (33.3%), moderately (33.3%), and severely dehydrated (22.2%). One calf was well hydrated and one calf was not assessed for hydration status. Six calves (85.7%) had acidosis as blood pH < 7.28 and, upon calculation, four of the six calves were found to have an elevated anion gap. Biochemical parameters were not available for three calves.

The mean ± SD of serum and fecal D- and L-concentrations are presented in Table 5.5. Upon review of individual calf data, D-lactic acidosis (serum D-lactate >3 mmol/L) was present in three diarrheic calves and two diarrheic calves had elevated serum L-lactate concentrations (>2 mmol/L). These samples were from calves admitted to the Western College of Veterinary Medicine at University of Saskatchewan. There was no significant linear regression between fecal L-lactate and serum L-lactate concentrations in this study, $P > 0.05$ (Figure 5.1). However, the linear regression between fecal D-lactate and serum D-lactate was statistically significant at $P < 0.0001$. Increases in fecal D-lactate levels will likely result in increasing serum D-lactate levels (Figure 5.2). There was no significant linear regression found between fecal D-lactate and fecal L-lactate concentrations (Figure 5.3).

Table 5.2 Clinical assessment scores of Quebec calves (n= 17) with diarrhea

	Physical appearance & behavior	Food & water intake	Rectal temperature	Character of Feces	Other
365 (A)	2	3	0	2	0
365 (B)	0	1	0	2	0
8283	0	0	0	2	0
8239	0	0	0	2	0
8232	0	0	0	2	2
8266	0	0	0	2	0
8242	0	0	0	2	0
8216	0	0	0	2	0
8320	0	0	0	2	0
8228	0	0	0	2	0
8315	0	0	0	2	2
8227	0	0	0	2	0
8329	0	0	0	2	0
8225	0	0	0	2	0
8323	0	0	0	2	0
8233	0	0	0	2	0
8255	0	0	0	2	0

Table 5.3 Clinical assessment scores of University of Saskatchewan calves (n= 9) with diarrhea

	Sex	Age (days)	Physical appearance & behavior	Food & water	Rectal temperature	Character of Feces	Other	Dehydration status	Treatment given
716883	F	6	3	3	3	3	reduced gut sounds	Severely dehydrated (12%)	Banamine & TMS
713537	M	4	3	3	3	3	calf died	Moderately dehydrated (7%)	Calf guard at 6h after birth
716885	F	6	1	1	0	1	n/a	Mildly dehydrated (5%)	Banamine & TMS
717019	M	38	3	3	3	3	euthanized, suspected viral enteritis (bovine coronavirus	Severely dehydrated (10%)	Calf Span (oral antibiotic), Borgal (antibacterial agent), tubing (milk/electrolytes)
716884	F	6	1	0	0	1	n/a	Mildly dehydrated (5%)	Banamine and TMS
714932	F	14	1	1	0	3	grade 3/6 heart murmur, wet umbilicus, very pale	Moderately dehydrated (8%)	Head Start at birth , Calf Lyte II, Resflor (antibiotic/anti-inflammatory)
714932-15	F	15	0	0	0	3	mucoid white nasal discharge	Mildly dehydrated	Fluid therapy IV and oral electrolytes
115	F	n/a	n/a	n/a	2	0	none	n/a	none
815	F	n/a	1	1	n/a	1	n/a	Not dehydrated	Kaopectate, Revibe (oral electrolyte powder)

F, female; M, male; TMS, Trace mineralized salt; n/a, not available

Table 5.4 Blood electrolyte concentrations in calves (n=23) with diarrhea

	Healthy Calves* mean ± SD	Quebec Samples (n=16) mean ± SD (range)	U of S Samples (n=7) mean ± SD (range)	All samples (n=23) mean ± SD
pH	7.36 ± 0.03	7.41 ± 0.07 (7.23-7.52)	7.21 ± 0.152 (6.91-7.37)	7.35 ± 0.138
Sodium (mmol/L)	135.6 ± 3.1	138.4 ± 5.66 (133-158)	138.5 ± 9.53 (128.6-153.4)	138.5 ± 6.83
Potassium (mmol/L)	5.0 ± 0.4	5.14 ± 0.842 (4.4-8.0)	4.70 ± 1.57 (3.19-7.4)	5.00 ± 1.09
Chloride (mmol/L)	102.4 ± 2.0	N/A	103.7 ± 6.05 (95-113)	N/A
Bicarbonate (mmol/L)	32.6 ± 2.1	28.2 ± 4.60 (18.2-32.8)	22.1 ± 11.1 (10.3-45.4)	26.4 ± 7.53
Anion Gap	5.6 ± 3.5	N/A	17.5 ± 4.88 (13.3-25.3)	N/A

*As blood gas measurements were not taken from healthy calves in this study, data for health calves were obtained from a previous study (Omole, 2001); N/A, not available.

Table 5.5 Serum and fecal D- and L-lactate concentrations in calves (n=26) with diarrhea

	Literature values of healthy calves*	Quebec Samples (n=16) mean ± SD (range)	U of S Samples (n=10) mean ± SD (range)	All samples (n=26) mean ± SD (range)
Serum				
D-Lactate, mmol/L	0.1-1.40	0.432 ± 0.477 (0.088-2.06)	1.95 ± 1.76 (0.041-4.29)	1.02 ± 1.35 (0.041-4.29)
L-Lactate, mmol/L	< 2.00	1.09 ± 0.311 (0.662-1.66)	1.69 ± 0.502 (1.06-2.56)	1.32 ± 0.476 (0.662-2.56)
Feces				
D-Lactate, mmol/L	1.2-24.4	4.15 ± 4.10 (0.374-17.3)	13.3 ± 13.0 (1.04-31.6)	7.65 ± 9.58 (0.374-31.6)
L-Lactate, mmol/L	0.9-23.6	9.04 ± 7.33 (0.560-24.8)	14.1 ± 11.7 (2.37-36.4)	11.0 ± 9.21 (.560-36.4)

* Lorenz et al., 2005; Abeysekara et al., 2007; Ewaschuk et al., 2003; Omole et al., 2001; Ewaschuk et al., 2004a; Shimomura & Sato, 2006; Sato & Koiwa, 2008

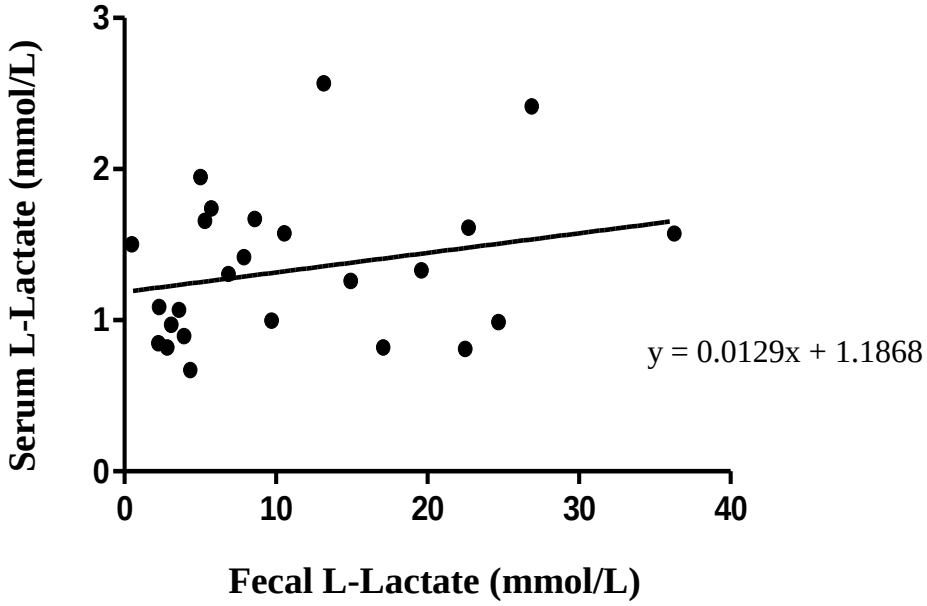

Figure 5.1 Linear regression between fecal L-lactate and serum L-lactate in diarrheic calves; n=25 (r^2 = 0.062; *P* > 0.05; ——— regression line).

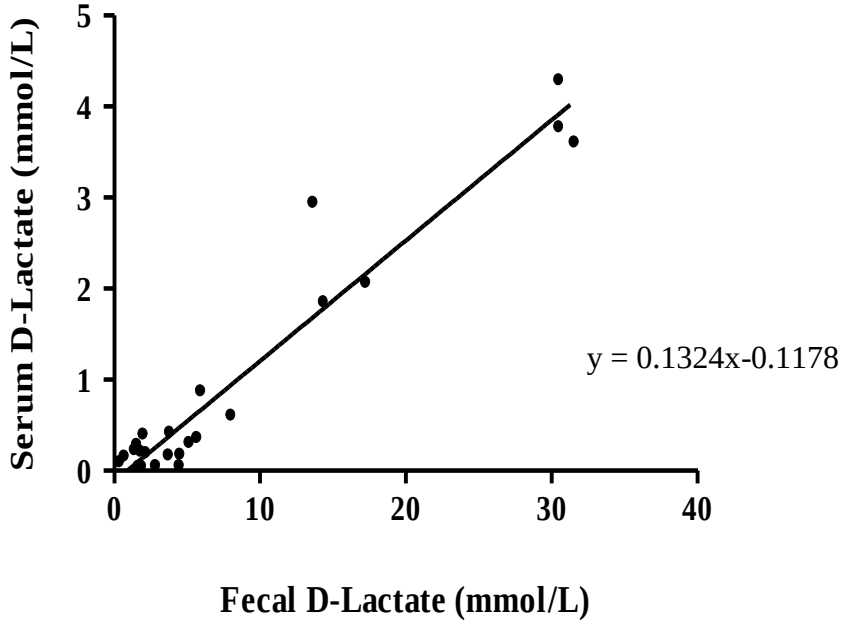

Figure 5.2 Linear regression between fecal D-lactate and serum D-lactate in diarrheic calves; n=25 (r^2 = 0.932; *P* < 0.0001; ——— regression line).

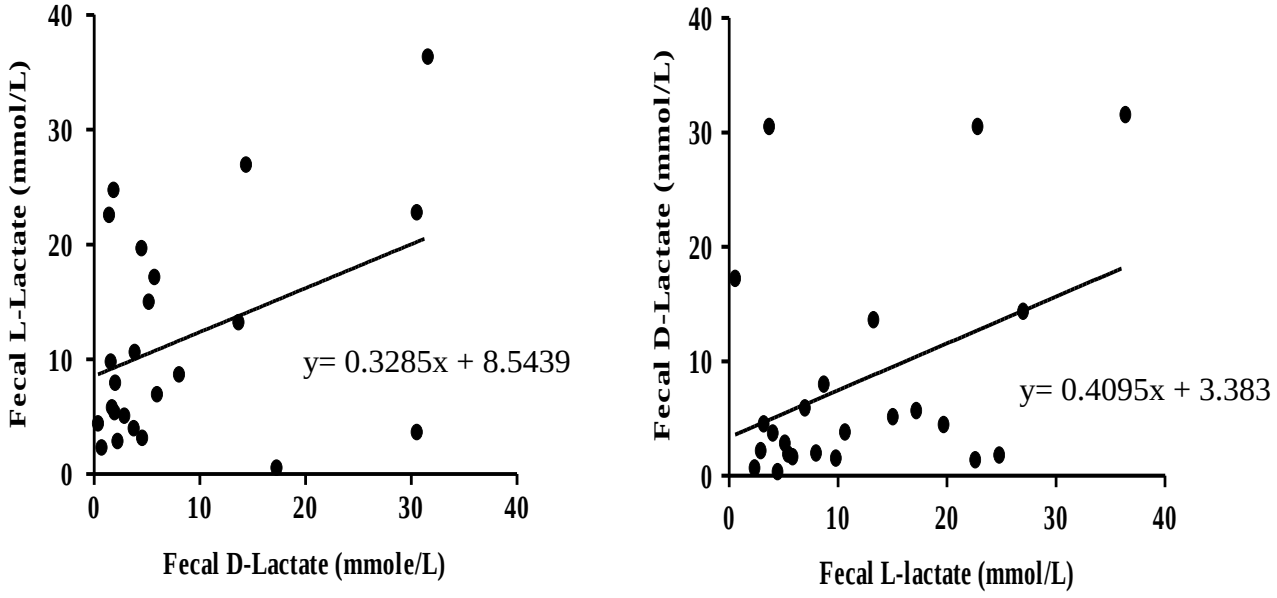

Figure 5.3 Linear regression between fecal D-lactate and fecal L-lactate in diarrheic calves; n=24 (r^2 = 0.157; *P* > 0.05; ——— regression line).

5.5 Discussion

Past research has shown that D-lactate is a major contributor to acidosis in diarrheic calves and elevated concentrations of fecal D-lactate found in these calves, when compared to healthy calves, suggest that the gastrointestinal tract is the site of excess D-lactate production (Omole et al., 2001; Ewaschuk et al., 2004a). Ewaschuk et al. (2004a) reported a significant positive correlation between fecal D-lactate concentrations and serum D-lactate concentrations in calves. This study's findings are consistent with Ewaschuk et al. (2004a) as fecal D-lactate concentrations were found to be significantly correlated with serum D-lactate concentrations and 93% of the variance is shared between fecal D-lactate and serum D-lactate. D-lactic acidosis has been reported in neonatal ruminants as a consequence of grain overfeeding, impaired ruminal milk fermentation, and neonatal diarrhea (Dunlop & Hammond, 1965; Omole et al., 2001; Ewaschuk et al., 2004a; Lorenz, 2004; Bleul et al., 2006; Lorenz & Lorch, 2009). In the gastrointestinal tract, D-lactate is produced by bacterial fermentation of carbohydrates in both animals and humans (Duncan et al., 2004; Halperin & Kamel, 1996). A loss in the ability to digest and absorb nutrients may result in malabsorption with abnormally high amounts of

carbohydrates being delivered to the large intestine. This results in increased gastrointestinal fermentation and, ultimately, increased production of D- and L-lactate (Peterson, 2005; Hove & Mortensen, 1995).

Malabsorption can occur in several clinical conditions, often resulting in diarrhea, and can be of nutritional origin (e.g. overfeeding, absence and/or deficiency in digestive enzymes) or due to infection of the gastrointestinal tract by enteric pathogens. In this study, samples from Quebec were collected from calves with diarrhea likely due to nutritional reasons (i.e. diet) while samples from University of Saskatchewan were collected from calves with diarrhea likely resulting from enteric pathogens. Some of the major enteric pathogens involved in neonatal calf diarrhea (e.g. *Cryptosporidium parvum)* disrupt the epithelial cells of the small intestine. As a result, villus atrophy leads to impaired nutrient digestion and absorption, which may lead to carbohydrate malabsorption (Smith, 2009; Lorenz, 2000). An increase in gastrointestinal fermentation creates a favourable environment (i.e. acidic) for the growth of acid-resistant lactate producing bacteria and as a result larger than normal amounts of D- and L-lactate are produced. When the rate of D-lactate absorption exceeds the body's ability to metabolize it, D-lactic acidosis may occur. In this study, the three cases of D-lactic acidosis were observed only in those calves with diarrhea likely caused by enteric pathogens.

This study showed no significant linear relationship between fecal L-lactate and serum L-lactate (Figure 5.1). These finding suggests that, in diarrheic calves, both D- and L-lactate are produced in the gastrointestinal tract however once however once absorbed into the systemic circulation, L-lactate is likely metabolized more efficiently when compared to D-lactate and does not result in a proportional increase in serum L-lactate concentration. In the liver, L-lactate is metabolized to pyruvate by the enzyme L-lactate dehydrogenase (Horton, 2002). D-lactate can also be metabolized to pyruvate by mitochondrial putative D-lactate dehydrogenase, with activity found highest in kidney and liver; however its uptake into the mitochondria is less efficient when compared to L-lactate (DeBari, et al., 2002; Flick & Konieczny, 2002).

D- and L-lactate may also be excreted by the kidneys; however, since both isomers use the same co-transport mechanism with sodium, it is proposed there is competition for reabsorption (Oh et. al, 1985). L-Lactate has been shown to be more efficiently reabsorbed by the renal tubules than D-lactate and this is thought to be a significant way of how the body reduces elevated blood D-lactate concentrations (Oh et.al, 1985; de Vrese et al., 1990; Uribarri,

1998). Oh et al. (1985) proposed that, since renal tubular reabsorption of lactate is reduced by increased urine volume, less D-lactate may be excreted during volume depletion and result in elevated blood D-lactate concentrations and/or D-lactic acidosis. In this study, three calves with D-lactic acidosis were clinically assessed as being dehydrated however urinary D-lactate concentrations were not determined.

From this study, fecal D-lactate production also appears to be independent of L-lactate production in the gastrointestinal tract as fecal D-lactate and fecal L-lactate concentrations were not found to be significantly correlated (Figure 5.3). This study also suggests that as diarrhea severity increases D-lactate concentrations also increase. Similar results were found in a study conducted at University of Saskatchewan in neonatal lambs (Abeysekara, 2009). It is evident from the clinical assessment that the calves from Quebec had less severe diarrhea when compared to the calves from the University of Saskatchewan clinic. Upon clinical assessment, diarrheic calves from Quebec, with the exception of one calf, appeared normal, bright, and alert with normal food and water intake, rectal temperature, and biochemical parameters. No clinical cases of D- and/or L-lactic acidosis were present. In Saskatchewan, two of the three calves with D-lactic acidosis were clinically assessed as physically recumbent and could not be roused, were not eating, hypothermic, and assessed as moderately to severely (7-12%) dehydrated.

A limitation of this study is that the fecal D-lactate concentrations on the higher end of the data set may be driving the correlation as there were few fecal samples with D-lactate concentrations greater than 10 mmol/L. As a result, predictions of blood D-lactate concentrations based on fecal D-lactate concentrations, at this time, are not possible. Abeysekara et al. (2009) reported a fecal threshold concentration for absorption of D-lactate into the blood of 10.2 mmol/L and 8.8 mmol/L in neonatal lambs and calves, respectively. Fecal and blood samples in this particular study were analyzed for D-lactate using the HPLC method. Due to the limit of quantitation, parameters that were not detectable by HPLC were assigned a value of 0 and parameters that were detectable but below the limit of quantification were assigned an arbitrary value. Using the enzymatic assay, this study was able to plot all absolute D-lactate concentrations. There is a clustering of fecal D-lactate concentrations below 10 mmol/L and these results are similar to those of Abeysekara (2009). In this study, however, the range of D-lactate concentrations, especially those >10 mmol/L, in the fecal samples was not large enough to conduct a biphasic (break-point) regression analysis. Researchers were unable to determine,

at this time, if a fecal threshold concentration exists for the absorption of D-lactate into the blood in calves with diarrhea.

Further investigation on a larger scale, with varying degrees of diarrhea severity, is warranted to examine whether or not the fecal D-lactate concentration threshold is confirmed at levels previously reported. If a threshold for the absorption of D-lactate into the blood exists in neonatal calves with diarrhea, fecal D-lactate concentrations may prove to be a useful biomarker to predict and possibly prevent D-lactic acidosis and its resulting neurological impairments. Future research could then investigate potential treatments that target gastrointestinal D-lactate producing bacteria. It may also be beneficial to determine whether or not a fecal D-lactate concentration threshold exists is different species as human children are also prone to acute diarrhea of infectious origins which result in acidosis. As thoroughly described in the previous chapter, it is unknown if D-lactate is a significant contributor to the acidosis seen in children with acute diarrhea and metabolic acidosis.

CHAPTER SIX
GENERAL DISCUSSION AND CONCLUSIONS

Diarrhea causes significant mortality and morbidity in several species worldwide. In animals, diarrheal diseases have a significant economic impact on livestock operations (USDA, 2008). In children, reduction of diarrhea-related mortality is essential towards achieving the Millennium Development Goal to "reduce by two-thirds, between 1990 and 2015, the mortality rate of children under five" (United Nations, 2002). Regardless of its cause, diarrheal diseases can result in clinical consequences including metabolic acidosis. D-Lactate has been identified as a significant contributor to acidosis in diarrheic calves (Kasari & Naylor, 1984; Omole et al., 2001; Ewaschuk et al., 2004a) and in humans with short bowel syndrome (Uribarri et al., 1998). Identifying elevated D-lactate concentrations in children with acute diarrhea in the absence of short bowel syndrome has been poorly examined. Further research of D-lactate is warranted given the significant impact of diarrheal diseases and potential role of D-lactate in metabolic acidosis.

Pre-analytical processing and storage methods have been shown to influence the stability of various solutes in blood samples following collection (Tuck et al., 2009). Past studies on lactate stability investigate changes in total lactate concentrations and do not determine the changes of each specific lactate isomer, D- and L-lactate. This led to the objectives of the current study as clinical symptoms reported in D-lactic acidosis including weakness, ataxia, impaired posture and behaviour, and, in severe cases, encephalopathy and coma (Kasari & Naylor, 1984; Kasari & Naylor, 1986; Lorenz, 2004; Lorenz et al., 2005; Forsyth et al., 1991; Grünert et al., 2010; Htyte et al., 2011; Jorens et al., 2004) differ from clinical symptoms seen in L-lactic acidosis. To be able to quantify both isomers, D- and L-lactate, in blood for proper clinical diagnosis and treatment is important. Therefore, to determine the stability of total, D-lactate, and L-lactate blood concentrations after prolonged contact of serum and plasma with blood cells and after immediate separation of serum and plasma via centrifugation, blood samples were obtained from eleven healthy calves (Chapter 3). Samples were stored at 4°C and analyzed for D- and L-lactate using an enzymatic assay at 1, 2, 4, 8, 12, 24, and 48 hours following collection.

D-lactate, and L-lactate concentrations in calf serum and plasma, stored at 4°C following separation from blood cells 0.5 hour post collection, were found to be stable up to 48 hours. In untreated serum samples when stored in contact with cells, D-lactate concentration was significantly higher, 82.3%, at 48 hours. L-lactate concentrations increased significantly in untreated and spiked serum and spiked plasma samples stored in contact with blood cells at 48 hours. Significant differences were also noted in D- and L-lactate concentrations when using a serum collection tube vs. plasma collection tube.

To our knowledge, this study was the first to investigate the stability of specific lactate isomers after prolonged contact of serum and plasma with blood cells and after immediate separation of serum and plasma via centrifugation. Based on the results of this study, to ensure a reliable measurement of D- and L- lactate concentrations, serum and/or plasma samples should be centrifuged as soon as possible following collection but can thereafter be stored at 4˚C for up to 48 h without noticeable changes in D- or L-lactate concentrations.

D-lactic acidosis has been documented in humans as a clinical complication of SBS. However, identifying elevated concentrations of D-lactate and D-lactic acidosis in children with acute diarrhea, in the absence of short bowel syndrome has been poorly examined. This warranted further investigation as infectious diarrhea has been shown to result in carbohydrate malabsorption and acidosis, similar to that found in SBS (Sack et al., 1982). To determine whether D-lactic acidosis occurs in children with diarrhea blood samples were obtained from nine children ≤ 5 years of age with acute diarrhea and suspected metabolic acidosis (Chapter 4). Samples were analyzed for pyruvate, D- and L-lactate using an enzymatic assay.

No clinical cases of D-lactic acidosis were found in this study's small population of children with acute diarrhea. Upon review of blood electrolytes and assessment of diarrhea severity, children selected for this study were not clinically acidodic and did not have extremely severe diarrhea. It is possible that the incidence of D-lactic acidosis in children with acute diarrhea may not be to the extent seen in diarrheic animals; however, this may be because children in developed countries are often assessed and treated for illness much sooner than that of animals. Work to conduct a similar study in a developing country, where more severe diarrhea is likely to occur, was initiated however, to date, no samples have been collected or analyzed. To adequately study D-lactic acidosis in children, it would be preferred to include a control group in order to determine a D-lactate concentration range in healthy children ≤ 5 years

of age and to include children with wide range of diarrhea severity. If D-lactic acidosis does occur in children with diarrhea, treatment strategies could be altered to target the D-lactate producing bacteria.

Although there were no clinical cases of D-lactic acidosis in this study, it is interesting that as a result of using an enzymatic assay, the researchers were able to detect D-lactate concentrations at lower levels when compared to a previously used HPLC analytical method (Ewaschuk et al., 2004b), and 66.7% of children in this study had a D-lactate concentration greater than that found in healthy adults (> 0.1 mmol/L). As children were 10 months to 3 years of age, this increase may be a result of immature metabolic mechanisms, however, the impact of diarrheal diseases on normal development and maturation rate of enzymes and transporters involved in D-lactate is unknown and may warrant further investigation. Future studies should also investigate fecal D-lactate concentrations as it is suggested, in animal studies, that a potential gastrointestinal threshold for the absorption of D-lactate into the blood exists and fecal D-lactate could prove to be a useful biomarker for D-lactic acidosis.

The final portion of this research aimed to examine a wide range of diarrhea severity to determine if a fecal D-lactate threshold exists at which D-lactate enters the systemic circulation in neonatal calves with diarrhea. Blood and fecal samples were obtained from 15 diarrheic calves (Chapter 5). Electrolytes were measured in the blood and all calves were clinically assessed. Blood and feces were analyzed for D- and L-lactate using an enzymatic assay. D-Lactic acidosis (> 3 mmol/L) was present in three diarrheic calves and two calves had elevated L-lactate concentrations. Fecal D-lactate concentrations were found to be positively correlated with serum D-lactate concentrations while fecal L-lactate and serum L-lactate was not found to be significantly correlated. This finding is supported by a past study conducted by Ewaschuk et al. (2004a). Both D- and L-lactate are produced via gastrointestinal fermentation in the large intestine. When there is a loss in the body's ability to digest and absorb nutrients, as observed in some cases of diarrhea, abnormally large amounts of carbohydrate are delivered to the colon resulting in increased colonic fermentation and increased production of D- and L-lactate. Both D- and L-lactate can be absorbed in the systemic circulation. Once in the blood, L-lactate is thought to be metabolized more efficiently than D-lactate. When the rate of D-lactate absorption exceeds the body's ability to metabolize it, D-lactate accumulates in the blood and can lead to D-lactic acidosis.

This study had few fecal D-lactate concentrations greater than 10 mmol/L therefore predictions of blood D-lactate based on fecal D-lactate are not possible. In a similar study, Abeysekara (2009) reported a fecal threshold concentration of 10.2 mmol/L and 8.8 mmol/L in neonatal lambs and calves, respectively but was only able to plot those D-lactate concentrations greater than 0.05 mmol/L using a HPLC method. Using an enzymatic assay, this study was able to plot all D-lactate concentrations and a clustering of D-lactate concentrations below 10 mmol/L was observed, similar to those results of Abeysekara (2009), however the range of D-lactate concentrations was not large enough to conduct a biphasic (breakpoint) regression analysis. Further study, with varying degrees of diarrhea severity, should continue to determine whether or not a fecal threshold exists at the levels previously reported. If a threshold exists, fecal D-lactate may prove to be a useful biomarker to predict and possibly prevent D-lactic acidosis and its neurological consequences.

In conclusion, this body of work continues to contribute to the knowledge of D-lactate and D-lactic acidosis in diarrhea. The effects of pre-analytical processing and storage of specific lactate isomers was determined and further investigation into a possible fecal D-lactate threshold was explored. Although D-lactic acidosis did not occur in children with acute diarrhea in this study, further study is warranted to investigate a wider range of diarrhea severity, the potential use of fecal D-lactate concentration as a biomarker for elevated blood D-lactate concentrations, and to further explore the mechanism and/or significance of why children with acute diarrhea appear to have a higher blood D-lactate concentrations when compared to healthy children.

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APPENDICES

Appendix A

Ethics Certificates

UNIVERSITY OF SASKATCHEWAN

Animal Research Ethics Board (AREB)

Certificate of Approval

PRINCIPAL INVESTIGATOR
Dr. Katharina Lohmann

DEPARTMENT/ORGANIZATION
Large Animal Clinical Sciences

ANIMAL USE PROTOCOL #
20060047

TITLE
Mechanisms of acidosis and acidosis treatment in calves.

SPONSORING AGENCIES
Natural Sciences Engineering Research Council (NSERC)

BIOSAFETY NUMBER
LAC-05, PAN-01

UNIFI FUND #
402833, 410312

APPROVAL DATE:
December 14, 2010

APPROVAL OF:
Renewal Animal Use Protocol

EXPIRY DATE:
December 14, 2011

Full Board Meeting ☒ AREB Subcommittee ☐ AREB Chair and University Veterinarian ☐ AREB Chair ☐

CERTIFICATION
The University of Saskatchewan Animal Research Ethics Board reviewed the above-named research project. The proposal was found to be acceptable on ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research project, and for ensuring that the authorized research is carried out according to the conditions outlined in the original protocol submitted for ethics review. This Certificate of Approval is valid for the above time period.

PROTOCOL MODIFICATIONS
Any modifications to this protocol must be approved by the UCACS AREB Chair prior to implementation, using the AUP Modification Form.

ONGOING REVIEW REQUIREMENTS
Research programs that extend beyond one year must receive annual review. For the annual renewal, an annual review form (and progress report) must be submitted to the AREB within one month of the current expiry date each year the study remains open, and upon study completion. Please refer to the Research Ethics Office website for further instructions.

Please send all correspondence to:
Research Ethics Office
University of Saskatchewan
Box 5000 RPO University, 1607-110 Gymnasium Place
Saskatoon SK S7N 4J8
Telephone: (306) 966-7925 Fax: (306) 966-2069 Email: ucacs.office@usask.ca

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval
Study Amendment

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Garth A. Bruce	Pediatrics	03-932

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT

Royal University Hospital
103 Hospital Drive
Saskatoon SK S7N 0W8

Irwin Nutrition Research Laboratory
Room 331, Thorvaldson Building
Saskatoon SK

SUB-INVESTIGATOR(S)
Gordon A. Zello, Jane Alcorn

STUDENT RESEARCHER(S)
Jennifer Wright

SPONSORING AGENCIES
UNIVERSITY OF SASKATCHEWAN - COLLEGE OF PHARMACY AND NUTRITION

TITLE
: D-Lactic Acidosis in Pediatric Diarrhea

APPROVAL OF	APPROVED ON	CURRENT EXPIRY DATE
Changes to chart review data collection Amended Subject Information and Consent Form (05-May-2010)	05-May-2010	11-Jun-2010

Delegated Review: ☒ Full Board Meeting: ☐

CERTIFICATION

The study is acceptable on scientific and ethical grounds. The Bio-REB considered the requirements of section 29 under the Health Information Protection Act (HIPA) and is satisfied that this study meets the privacy considerations outlined therein. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face) meeting. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://www.usask.ca/research/ethics_review.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. This approval and the views of this REB have been documented in writing.

Please send all correspondence to:

Research Ethics Office
University of Saskatchewan
Box 5000 RPO University
1607-110 Gymnasium Place
Saskatoon SK S7N 4J8

UNIVERSITY OF SASKATCHEWAN Biomedical Research Ethics Board (Bio-REB)

Certificate of Re-Approval

PRINCIPAL INVESTIGATOR	DEPARTMENT	BMC #
Garth A. Bruce	Pediatrics	03-932

INSTITUTION (S) WHERE RESEARCH WILL BE CARRIED OUT

Irwin Nutrition Research Laboratory
Room 331, Thorvaldson Building
Saskatoon SK

Royal University Hospital
103 Hospital Drive
Saskatoon SK S7N 0W8

SUB-INVESTIGATOR(S)

Gordon A. Zello, Jane Alcorn

STUDENT RESEARCHER(S)

Jennifer Wright

SPONSORING AGENCIES

UNIVERSITY OF SASKATCHEWAN - COLLEGE OF PHARMACY AND NUTRITION

TITLE:

D-Lactic Acidosis in Pediatric Diarrhea

RE-APPROVED ON	EXPIRY DATE
17-May-2010	11-Jun-2011

Full Board Meeting ☐

Delegated Review ☑

CERTIFICATION

The study is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This re-approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face meeting. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://www.usask.ca/research/ethics_review/.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. This re-approval and the views of this REB have been documented in writing.

Please send all correspondence to:

Research Ethics Office
University of Saskatchewan
Box 5000 RPO University
1607 - 110 Gymnasium Pl
Saskatoon, SK S7N 5C8

Associate Vice-President Research – Health
(University of Saskatchewan)
Vice-President Research and Innovation
(Saskatoon Health Region)
Room B527 Health Sciences Building
University of Saskatchewan
107 Wiggins Road
Saskatoon, SK S7N 5E5
Phone: (306) 966-8745

DATE: October 8, 2009

TO: Dr. G. Bruce
Dept. of Pediatrics
RUH

FROM:

RE: **RESEARCH PROJECT ETHICS COMMITTEE (EC)#: 2003-932**
PROJECT NAME: D-lactic acidosis in pediatric diarrhea
PROTOCOL #: NA

Saskatoon Health Region is pleased to provide you with operational approval of the above-mentioned research project.

Kindly inform us when the data collection phase of the research project is completed. We would also appreciate receiving a copy of any publications related to this research. As well, any publications or presentations that result from this research should include a statement acknowledging the assistance of Saskatoon Health Region.

We wish you every success with your project. If you have any questions, please feel welcome to contact Shawna Weeks at 655-1442 or email shawna.weeks@saskatoonhealthregion.ca

Catalyzing Health Research and Innovation Together

Associate Vice-President Research – Health
(University of Saskatchewan)
Vice-President Research and Innovation
(Saskatoon Health Region)
Room 247-111 Research Drive
Atrium Building, Innovation Place
Saskatoon, SK S7N 3R2
Phone: (306) 966-8745

DATE: November 22, 2010

TO: Dr. Garth Bruce
Dept. of Pediatrics
Royal University Hospital

FROM:

RE: **RESEARCH ETHICS BOARD (REB) #: 203-932**
PROJECT NAME: D-Lactic Acidosis in Pediatric Diarrhea
PROTOCOL #: N/A

Saskatoon Health Region is pleased to provide you with amended operational approval of the above-mentioned research project to include Acute Care Pediatrics.

Kindly inform us when the data collection phase of the research project is completed. We would also appreciate receiving a copy of any publications related to this research. As well, any publications or presentations that result from this research should include a statement acknowledging the assistance of Saskatoon Health Region.

We wish you every success with your project. If you have any questions, please feel welcome to contact Shawna Weeks at 655-1442 or email shawna.weeks@saskatoonhealthregion.ca

Catalyzing Health Research and Innovation Together

UNIVERSITY OF SASKATCHEWAN

Animal Research Ethics Board (AREB)

Certificate of Approval

PRINCIPAL INVESTIGATOR	DEPARTMENT/ORGANIZATION	ANIMAL USE PROTOCOL #
Dr Gordon Zello	Pharmacy and Nutrition	20090135

TITLE
D-Lactic acidosis in neonatal diarrhea

APPROVAL DATE:	APPROVAL OF:	EXPIRY DATE:
October 27, 2009	New Animal Use Protocol	October 27, 2010

Full Board Meeting ☒ AREB Subcommittee ☐ AREB Chair and University Veterinarian ☐ AREB Chair ☐

CERTIFICATION
The University of Saskatchewan Animal Research Ethics Board reviewed the above-named research project. The proposal was found to be acceptable on ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research project, and for ensuring that the authorized research is carried out according to the conditions outlined in the original protocol submitted for ethics review. This Certificate of Approval is valid for the above time period.

PROTOCOL MODIFICATIONS
Any modifications to this protocol must be approved by the UCACS AREB Chair prior to implementation, using the AUP Modification Form.

ONGOING REVIEW REQUIREMENTS
Research programs that extend beyond one year must receive annual review. For the annual renewal, an annual review form (and progress report) must be submitted to the AREB within one month of the current expiry date each year the study remains open, and upon study completion. Please refer to the Research Ethics Office website for further instructions.

PLEASE NOTE THE FOLLOWING:
To minimize risks associated with the shipment of samples to the University of Saskatchewan, please ensure that your colleague from Hawassa University packages and ships the blood and fecal samples according to international transportation guidelines.

Please send all correspondence to:
Research Ethics Office
University of Saskatchewan
Box 5000 RPO University, 1607-110 Gymnasium Place
Saskatoon SK S7N 4J8
Telephone: (306) 966-7928 Fax: (306) 966-2069 Email: ucacs.office@usask.ca

Appendix B

D-Lactic Acidosis in Neonatal Diarrhea (Awassa, Ethiopia)

INTRODUCTION

The College of Agriculture and Bioresources and College of Pharmacy and Nutrition at the University of Saskatchewan have developed a partnership with Hawassa University (HU) in Awassa, Ethiopia in building and improving the capacity of the HU curriculum development, teaching, graduate student supervision and research collaboration. Ethiopia is an agrarian country where small-scale farmers dominate agricultural production. Food insecurity and chronic malnutrition is a persistent problem and significantly impacts the health and wellbeing of households (Jufare, 2008). A recent survey conducted by Arcand et al. (2009) identified several potential areas of research for graduate students including livestock and human health. The Global Partner II Program provided the UofS student researcher the opportunity to travel to Awassa, Ethiopia to continue strengthening the research collaboration developed between HU and UofS and to work jointly with HU graduate students on nutritional research.

Diarrhea in children and neonatal animals continues to be a leading cause of morbidity and mortality in developing countries. Children are more likely to have a higher number of diarrheal episodes, severe episodes with dehydration, and higher death rate compared to those children living in countries living in a middle-or high-income country (O'Ryan, Prado & Pickering, 2005). In Ethiopia, an estimated 73,341 child deaths were due to diarrhea in 2008 (Black et al., 2010). In neonatal calves, diarrhea related morbidity and death have a significant impact on the wellbeing of Ethiopian farmers as they are dependent on livestock for food, income, draught power for crop production and financial security (Wudu, Kelay, Mekonnen & Tesfu, 2008; Randolph et al., 2007). Metabolic acidosis is a clinical complication of diarrhea in children and neonatal animals. As previously described, D-lactate, a physiological isomer of L-lactate, was found to be a significant contributor to acidosis in diarrheic calves (Kasari & Naylor, 1984; Omole, Nappert, Naylor & Zello, 2001; Ewaschuk, Naylor, Palmer, Whiting & Zello, 2004). It is not known if D-lactic acidosis occurs in neonatal animals with diarrhea in Ethiopia. A previously discussed in Chapter 3, it is also not known if D-lactic acidosis occurs in children, in the absence of short bowel syndrome, with acute diarrhea. Clinical cases of pediatric diarrhea in Saskatchewan where health care, for the most part, is readily accessible may not be severe enough to cause an overproduction of D-lactate in the gastrointestinal tract and D-lactic acidosis. By conducting a parallel study in Ethiopia, where 85% of the population lives in rural areas and

more severe and prolonged diarrhea is likely to occur, a larger sample size can be obtained with a wider range of diarrhea severity.

If this study shows that children and/or neonatal animals with diarrhea have D-lactic acidosis, future research may investigate treatments to target the formation of colonic lactate, specifically the D-lactate producing bacteria, as opposed, or in conjunction with, the buffering or oral replacement therapies currently used. As use of medicinal remedies (e.g., antibiotics) in developing countries is costly and not readily available, and due the increasing concerns surrounding antibiotic resistance, further research could investigate the treatment and prevention of diarrhea with natural sources of food (e.g., pre and probiotics).

The objectives of this research project were:

- To engage and support a nutrition graduate student from HU in a parallel project to the D-lactic acidosis studies conducted in Saskatoon, Saskatchewan:
    - To determine if D-lactic acidosis occurs in children (≤ 5 years of age) who come to Awassa Referral Hospital with acute diarrhea and metabolic acidosis in Awassa, Ethiopia
    - To determine if D-lactic acidosis occurs in neonatal animals in Awassa, Ethiopia
- To collaborate with Hawassa University (HU) including the Masters of Human Nutrition [MAHN] program, Awassa Referral Hospital, and Department of Veterinary Medicine, in carrying out nutrition research

MATERIALS AND METHOD

Study Design

Human Study

For the investigation of D-lactic acidosis in human children, this study is designed to parallel that of "D-Lactic Acidosis in Pediatric Diarrhea" as previously described in Chapter 3. Children (≤ 5 years of age) with acute diarrhea, defined as three or more profuse or watery stools per day, would be eligible for this study. Once parental or legal guardian consent was obtained, blood and fecal samples would be collected from each study participant and analyzed for D-, L-lactate and pyruvate concentrations. Again, diarrhea would have to be severe enough to warrant routine clinical investigation of blood electrolytes.

Animal Study

In May 2010, nine goat kids, less than 35 days of age, were selected for blood and fecal sampling based on clinical signs of diarrhea from a goat and sheep research farm located at Hawassa University. Control samples (blood and fecal) were also obtained from nine healthy goat kids from the same research farm. Blood and fecal samples were also obtained from one diarrheic neonatal calf at a nearby village veterinarian clinic. Animals were selected for this study on the basis of having clinical signs of diarrhea. All animals were assigned a clinical depression score and assessed for dehydration and diarrhea type (Appendix F). At the time of this study, there was no animal ethics board at HU however was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use (Appendix B).

Sample Collection and Analysis

Human Study

In Ethiopia, all health research involving humans must be approved by the National Health Research Ethics Committee. As discussed in 2009 with our HU partners and HU graduate student, a research proposal and ethics application was to be composed by the HU graduate student with guidance from the UofS researchers. A study proposal and study protocol was drafted and reviewed by UofS researchers however this document and ethics application was not submitted to the National Health Research Ethics Committee in Ethiopia before travelling back in May, 2010. Since human ethic approval was not granted upon my return to Awassa, no further work could be carried out for the human D-lactate project. Due to unforeseen circumstances, a research proposal was not submitted to the Ethics Committee therefore no blood and/or fecal samples have collected to date. The proposed study protocol prepared by the HU graduate student can be found in Appendix C.

Animal Study

Jugular vein blood samples, approximately 5-10mL, were collected from each animal and pH was determined immediately (Accumet basic pH meter; Fishers Scientific, Schwerte, Germany). The blood was then allowed to coagulate at room temperature for 20 minutes, centrifuged, and serum drawn off and stored at -20˚C. Approximately 20 g of feces was

collected from each animal and mixed with 15 mL Thiomerosal, a bacteriostatic agent, and frozen at -20˚C until analysis (Appendix C).

RESULTS

Two goat kids with diarrhea had blood pH < 7.28, which is indicative of acidosis. Upon clinical assessment, one kid with indicators of acidosis had no suck. The anion gap, which is often used to diagnosis metabolic acidosis, could not be calculated as researchers did not have access to a blood gas and electrolyte analyzer. Upon clinical assessment, the diarrheic calf had a weak menace but demonstrated normal suck, palpebral and tactile responses, and ability to stand. The blood pH of this calf was not indicative of acidosis.

STUDY PROGRESS

Serum and fecal samples were to be analyzed for D- and L-lactate using enzymatic assay kits (Biovision, Mountain View, CA). The analysis for this project required the use of a microplate reader, which was determined to be available at HU. However, the UofS student researcher was unaware that this piece of equipment had never been used prior to this study and no one within the department had any knowledge of its use. Due to some difficulties in set-up (i.e. misplacement of software) and technological difficulties (i.e. unable to obtain required optical density for microplate) samples could not be analyzed while the UofS student researcher was in Awassa, Ethiopia however technical skills and instruction on the use of the analytical kits was provided to the HU graduate student recruited for the study. Sample collection and analysis was to continue once the UofS student researcher left Ethiopia however, to date; no additional samples have been collected and/or analyzed.

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Appendix C

Study Protocols

PROTOCOL
D-Lactic Acidosis in Pediatric Diarrhea

Purpose: To determine the prevalence of D-lactic acidosis (i.e. from colonic fermentation) in pediatric diarrhea

Inclusion Criteria: Any pediatric patient (≤ 5 years of age) with acute diarrhea who will have serum electrolytes analysed

- **Physician(s) and/or researcher is to obtain consent from parents/legal guardian:**
  (Note: x2 consent forms are included in each study package: the signed copy is to be saved on the ward for researcher and the other copy given to the parent/legal guardian)

  - **Please have the first serum that is obtained for routine electrolyte analysis saved, by indicating "Save for study #03-932" on clinical chemistry requisition**

  - **Stamp blank Ziplock bag label with RUH #**

  - **Collect soiled diaper (must contain stool)**
    - **bag may be given to parent/legal guardian for collection while in hospital**

  - **Place diaper in Ziploc bag (provided), complete label on bag and place in freezer designated for study**

- **Please contact: for fecal sample pick up. Please leave a message if there is no answer.**

Thank you!

For more information or questions:

Jennifer Wright
College of Pharmacy and Nutrition
jennifer.wright@usask.ca

PROTOCOL
D-Lactic Acidosis in Pediatric Diarrhea

Purpose: To determine the prevalence of D-lactic acidosis (i.e. from colonic fermentation) in pediatric diarrhea

Inclusion Criteria: Any pediatric patient (≤ 5 years of age) with acute diarrhea who will have serum electrolytes analysed

- **Physician(s) and/or researcher is to obtain consent from parents/legal guardian: (Note: x2 consent forms are included in each study package: the signed copy is to be saved on the ward for researcher and the other copy given to the parent/legal guardian)**

****NOTE: Researcher is available at *any time* to complete the consent and collection process. Please call _ _ _ ___ (leave message if no answer)**

  - **Please have the first serum that is obtained for routine electrolyte analysis saved, by indicating "Save for study #03-932" on clinical chemistry requisition**
  - **Stamp blank Ziplock bag label with RUH #**
  - **Collect soiled diaper (must contain stool)**
    - **bag may be given to parent/legal guardian for collection while in hospital**
  - **Place diaper in Ziploc bag (provided), complete label on bag and place in freezer designated for study**
  - **Complete Diarrhea Severity Tool (attached to consent form) with parent(s) and/or legal guardian**

- **Please contact: for fecal sample pick up. Please leave a message if there is no answer.**

Thank you!

For more information or questions:
Jennifer Wright
College of Pharmacy and Nutrition
jennifer.wright@usask.ca

Comparison of Fecal and Serum D-lactate concentration in diarrheic calves

Katharina Lohmann, July 2010

Goal: To evaluate the relationship between fecal and serum D-lactate concentration in diarrheic calves.

Objectives:

1. To measure D-lactate concentration in serum and feces obtained simultaneously from diarrheic calves prior to treatment. Follow-up samples 24 hours later can also be collected if diarrhea persists.

2. To measure fecal dry matter content in diarrheic calves.

Materials:

Gloves
Fecal collection cups
Fecal containers suitable for storage in liquid nitrogen/ at -80 °C
Blood collection tubes (no additive)
Syringes and needles or vacutainer set-up
Centrifuge
Microfuge tubes or other suitable tubes for serum storage at -80 °C
Liquid nitrogen (N_2)
-80 °C freezer
Dry ice for shipment

Calves:

Calves less than 4 weeks of age with spontaneously occurring diarrhea are included. Samples should be collected prior to treatment; follow-up samples 24 hours later are collected if diarrhea persists.

Collection protocol:

1. Label the blood collection tube, serum storage tube, fecal collection cup and fecal storage container with the calf number, date and time of collection.

2. Fill out a calf record sheet including the clinical assessment score.

3. Collect a blood sample from the jugular vein or tail vein into a serum tube (5-10 ml). Allow sample to clot at room temperature for 20 minutes.

4. Wearing gloves, collect a fecal sample (10-15 grams) into a collection cup. If necessary, transfer the sample into a clean container prior to freezing.

5. Immediately after collection, snap freeze fecal sample in liquid N_2. Ensure the container is properly sealed to avoid leakage or entry of liquid N2 into the sample.

6. Once the blood sample is clotted, centrifuge for 15 minutes at approximately 3000 rpm (1500 g). Transfer serum into the serum storage tube and freeze at -80 °C.

Shipping:

Ship samples on dry ice to avoid thawing. Ship to:

Katharina Lohmann
Department of Large Animal Clinical Sciences
WCVM, University of Saskatchewan
52 Campus Drive
Saskatoon, SK S7N5B4
Phone (306) 966 7157
Fax (306) 966 7159
Email k.lohmann@usask.ca

PROTOCOL

D-Lactic Acidosis in Pediatric Diarrhea

Investigators:
Principal Investigator: Dr. Gordon Zello, College of Pharmacy & Nutrition, University of Saskatchewan, Canada
Co-investigator: Dr. Yfru Beyene, College of Health Sciences, Hawassa University, Awassa, Ethiopia
Graduate Students: Biniam Amare, Hawassa University, Ethiopia
Jennifer Wright, University of Saskatchewan, Canada
Contacts: Canada- Jennifer Wright
jennifer.wright@usask.ca
Ethiopia- Biniam Amare
biniam.amare@gmail.com

Purpose: To determine the prevalence of D-lactic acidosis in pediatric diarrhea

- **Inclusion Criteria:** Any pediatric patient (< 5 years of age) with clinical signs of diarrhea, defined as 3 or more profuse or watery stools per day
- **Please contact: to notify possible study participant.**
- **Once consent is obtained by researcher:**
- **Please have serum that is obtained for normal investigation of the patient based on the inclusion criteria, save it by indicating "Save for D-lactate study"**
- **Take 50g feces and place it in a white plastic jars, label on jar with subject #, and place in freezer**
- **Please fill out form provided**

Thank you!

For more information or questions:

Biniam Amare
Institute of Nutrition, Food Science and Technology,
Hawassa University
P.O.Box 05
Awassa, Ethiopia
biniam.amare@gmail.com

D-Lactic Acidosis in Pediatric Diarrhea Collection Form

Subject	**Stool** Saved ? If so, check box	**Serum** Saved? If so, check box	**Mark level of severity ****						
			Duration of diarrhea	Maximum number of diarrhea	Duration of vomiting	Maximum number of vomiting episodes	Temperature (°C)	Dehydration	Treatment
1									
2									
3									
4									
5									
6									
7									

** Refer to the Clerk or Vesikari severity scoring scales given below

Annex: The Clark and Vesikari severity scoring scales for the evaluation of gastroenteritis in children (one of the two scales will be selected based on the capacity of the hospital)

The physician, nursing staff, and/or research assistant will observe and classify the severity of diarrhea based on either of the following severity scoring scale.

Clark	**Point value**		
	1	**2**	**3**
Diarrhea			
Number of stools/per	2-4	5-7	≥8
Duration in days	1-4	5-7	≥ 8
Vomiting			
Number of emeses/day	1-3	4-6	≥ 7
Duration in days	2	3-5	≥ 6
Rectal temperature			
Temperature (°C)	38.1-38.2	38.3-38.7	≥ 38.8
Duration in days	1-2	3-4	≥ 5
Behavioural symptoms/signs			
Description	Irritable/less playful	Lethargic/listless	Seizure
Duration in days	1-2	3-4	≥ 5

Vesikari		Point value		
	0	1	2	3
Duration of diarrhea (days)	-	1-4	5	≥ 6
Maximum number of diarrhea stools/24 hr	-	1-3	4-5	≥ 6
Duration of vomiting (days)	-	1	2	≥ 3
Maximum number of vomiting episodes/24hr	-	1	2-4	≥ 5
Temperature (^{o}C)	-	37.1-38.4	38.5-38.9	≥ 39.0
Dehydration	-	-	Mild	Moderate to severe
Treatment	None	Oral Rehydration	Hospitalization	-

PROCEDURE FOR SAMPLE ANALYSIS

1) Plasma or serum preparation.

   a. Measure the pH of the samples.

   b. Centrifuge blood samples at ~1500-2000 X g for 10-15 min at room temperature.

      i. If it is serum (blood samples collected in tubes without anticoagulant), let the sample stand for 30 minutes before you centrifuge the samples.

   c. Remove the plasma with a transfer pipette, being careful not to disturb the WBCs.

   d. Put the collected plasma samples in -20 degree for future analysis.

Thank you! Any further question regarding protocol for study please contact Biniam Amare

Protocol for D-Lactic Acidosis in Animal Neonatal Diarrhea

Investigators:

Principal Investigator: Dr. Gordon Zello, College of Pharmacy & Nutrition, University of Saskatchewan, Canada

Co-investigator: Dr. Kassahun Asmare, Veterinary Medicine, Hawassa University, Ethiopia

Graduate Students: Biniam Amare, Hawassa University, Ethiopia
Jennifer Wright, University of Saskatchewan, Canada

Contacts: Canada- Jennifer Wright
jennifer.wright@usask.ca

Ethiopia- Biniam Amare
biniam.amare@gmail.com

Title: **Prevalence of D-lactic acidosis in neonatal animals**

Objective: To determine if D-lactic acidosis occurs in diarrheic neonatal animals in Awassa, Ethiopia

Methodology:

****Phone Biniam at) and let Biniam know that a sample has been taken. Leave a message if there is no answer.****

Study Design

- Neonatal animal inclusion criteria: clinical signs of diarrhea, defined as 3 or more profuse or watery stools per day

- Sampling (serum and feces) of neonatal animals at University Hawassa, Ethiopia will be conducted throughout the year. We hope to ensure the coverage of at least 15 cases per species over the course of one year.

- Blood (5-10ml) and fecal (approximately 50g [solid] or 100ml [liquid]) samples will be collected from diarrheic neonatal animals.
    - Blood will be analyzed for pH and allowed to clot. Serum will be drawn off for determination of organic acid concentration (D-,L- and pyruvate) at Department of Veterinary Medicine at Hawassa University.
    - Fecal samples to be frozen as soon as possible after collection

- In order to have a control, whenever samples are collected from diarrheic cases samples

(blood and feces) will be taken from a healthy neonatal animal of the same age and species.

- Serum and fecal samples will be stored at -20°C until analysis.

- Serum and feces will be analyzed for D-, L-lactate, and pyruvate via colorimetric assay kits (Biovision #K667-100, K607-100 and K609-100 respectively) at Hawassa University.

Procedure

Upon finding a potential diarrheic neonatal animal appropriate for study:

- Record animal #, species, age & sex on **Record Sheet A** (attached)

- Assess clinical signs according to scale provided and record depression score for each animal upon admission on **Record Sheet B**

- Assess dehydration according to **Sheet C** and record % dehydration on **Record Sheet A**

- Assess diarrhea type according to **Sheet D** and record diarrhea type on **Record Sheet A**

- Address both questions on the bottom of **Record Sheet A**

- Take initial samples from animal (see instructions below)
  - Blood for PH and drawing off serum (5-10ml)
  - Feces (~20 g)-cream coloured containers

- If possible, obtain sample from healthy neonatal animal of same species, record animal #, species, age, & sex
  - indicate on record sheet/labels if sample healthy (control) or diarrheic

- All samples should be well labelled with date, animal #, contents, name of (labels provided)

- All samples to be analyzed at Hawassa University (serum & feces) should be stored in freezer (-20°C)

Sample Collection

Blood

- A blood sample will be collected from the jugular vein of the neonatal animal in a collection tube and pH will be determined

- Serum organic acid concentration will be collected into a non-heparinized (containing no anticoagulant) collection tube.
    - The blood will be allowed to coagulate at room temperature for 20 min, centrifuged, and serum drawn off
    - Transfer serum to clean vial, label with date and case number
    - Serum samples will be stored in a freezer (-20°C) until analysis.

Feces

- Fecal samples (20g) will be collected into a cream colored plastic container (provided) containing Thiomerosal
    - Collected feces should be dissolved in 20 ml of 1% sodium ethylmercuric thiosalicylate (thiomerosal; ICN Biomedicals Inc., Aurora, Ohio, USA) solution (a bacteriostatic agent) to minimize bacterial growth.

- Thiomersal is a toxic substance, containing a small amount of mercury. Therefore you should not spill or contact thiomersal with exposed skin. Always use gloves (provided) when sampling or handling.

- Fecal samples to be stored in freezer (-20°C)

- Samples to be analyzed via assay kits as previously described at Hawassa University.

Thank you! Any further question regarding protocol for study please contact Biniam Amare

Appendix D

Consent Forms

SUBJECT INFORMATION AND CONSENT FORM

STUDY TITLE: D-Lactic Acidosis in Pediatric Diarrhea

PRINCIPAL INVESTIGATOR: Dr. Garth Bruce

SUB-INVESTIGATORS and/or STUDENT RESEARCHERS: Dr. G.A. Zello, Dr. J. Alcorn, Jennifer Wright, M.Sc. (Candidate)

FUNDING AGENCY: Natural Sciences and Engineering Research Council of Canada (NSERC)

CONTACT NUMBER: Jennifer Wright, M.Sc. Candidate (306) 371-7115

INTRODUCTION

Your child is being asked to take part in this research because he/she has been admitted to the Royal University Hospital for acute gastroenteritis and is experiencing diarrhea (2 or more profuse or watery stools per day). Participation is voluntary and it is up to you if you wish your child enrolled in this study. If you do decide that your child will take part in this study, you are still free to withdraw your child at any time and without giving any reasons for your decision. If you do not wish your child to participate, your child will not lose the benefit of any medical care to which your child is entitled or is presently receiving.

WHO IS CONDUCTING THE STUDY?

This study is being conducted by the College of Pharmacy and Nutrition at the University of Saskatchewan. Funding for this study is provided by the Natural Sciences and Engineering Research Council of Canada (NSERC). The investigators or staff will not receive any direct financial benefit from conducting this study, other than their regular salary.

WHY IS THIS STUDY BEING DONE?

The purpose of this study is to investigate the prevalence of D-lactic acidosis in children with diarrhea. Diarrhea results in changes in blood pH. Blood pH is normally neutral but in some children with diarrhea it becomes more acidic. Research originating from Dr. Zello's research group at the University of Saskatchewan showed that in animals the increase in blood acidity is due to a compound called D-lactic acid produced by bacteria present in the gut. It is not known if it is the same in humans. The purpose of the study is to evaluate if the type and severity of diarrhea in children is associated with the presence of D-lactic acid.

WHAT DOES THE STUDY INVOLVE?

We are requesting your permission to use leftover blood samples that are normally discarded after routine diagnostic tests have been done and to collect a sample of your child feces. **Your child medical care will not be delayed or affected in any way.** If you agree, the researcher will ask you 6 questions to help rate the severity of your child's diarrhea and a small amount of expelled feces will be collected. Blood samples that are ordered as part of routine care that are no longer needed for diagnostic purposes will be saved. Blood and feces samples will be transported to the Irwin Nutrition Research Laboratory at the University of Saskatchewan and analyzed for D- and L-lactic acid content. Your child's medical chart will also be reviewed for medical history, current medications and the results of the routine blood tests to

compare with the lactic acid levels measured as part of this study. Blood samples will be destroyed at the end of the study.

WHAT ARE THE RISKS AND BENEFITS OF PARTICIPATING IN THIS STUDY?
There are no risks to your child from participation in this study. There will not be any direct benefits to your child. It is hoped the information gained from this study can be used in the future to benefit other children with a similar condition.

WHAT HAPPENS IF I DECIDE TO WITHDRAW?
Your child's participation in this research is voluntary. If you choose to enter your child in the study and then decide to withdraw later, you may ask that the samples be destroyed by contacting the study doctor. All data collected about you during your enrolment will be retained for analysis.

WHAT WILL THE STUDY COST ME?
You will not be charged for any research-related procedures. You will not be paid for participating in this study. You will not receive any compensation, or financial benefits for being in this study, or as a result of data obtained from research conducted under this study.

WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?
In Saskatchewan, *the Health Information Protection Act (HIPA)* protects the privacy of your personal health information. Your child's confidentiality will be respected. No information that discloses your child's identity will be released or published without your specific consent to the disclosure. However, research records and medical records identifying your child may be inspected in the presence of the Investigator and the University of Saskatchewan Research Ethics Board for the purpose of monitoring the research. However, no records, which identify your child by name or initials, will be attached to any information. The results of this study may be presented in a scientific meeting or published, but your child's identity will not be disclosed.

WHO DO I CONTACT IF I HAVE QUESTIONS ABOUT THE STUDY?
If you have any questions or desire further information about this study before or during participation, you can contact anyone of the researchers: Jennifer Wright (306) 371-7115, Dr. G Zello (306) 966-5825, Dr. J. Alcorn (306) 966-6365, Dr. G. Bruce (306) 966-8117.

If you have any concerns about your child's rights as a research subject and/or your child's experiences while participating in this study, contact the Chair of the Biomedical Research Ethics Board (Bio-REB) of the University of Saskatchewan, at 306-966-4053. The Research Ethics Board is a group of individuals (scientists, physicians, ethicists, lawyers and members of the community) that provide an independent review of human research studies. This study has been reviewed and approved on ethical grounds by the Biomedical Research Ethics Board (Bio-REB) of the University of Saskatchewan.

CONSENT TO PARTICIPATE
I agree to have my child participate in this study. I give permission to the use and disclosure of my child's de-identified personal health information collected for the research purposes described in this form. I understand that by signing this document I do not waive any of my child's legal rights. I will be given a signed copy of this consent form.

Printed name of parent/legal guardian:	Signature	Date
Printed name of person obtaining consent:	Signature	Date

WESTERN COLLEGE OF VETERINARY MEDICINE
UNIVERSITY OF SASKATCHEWAN

REQUEST FOR OWNER CONSENT TO PARTICIPATION IN RESEARCH

Title of Proposal: **Mechanisms of acidosis and acidosis treatment in calves**

Principle Investigators and Co Investigators:
Katharina L. Lohmann, Med Vet, PhD, DACVIM
Associate Professor, Department of Large Animal Clinical Science, WCVM
Phone: (306) 966 7178

Gordon A. Zello, B.Sc., M.Sc., Ph.D.
Professor of Nutrition and Dietetics, College of Pharmacy and Nutrition, University of Saskatchewan

Funding Source: National Sciences and Engineering Research Council (NSERC)

PLEASE READ CAREFULLY BEFORE SIGNING

You have been asked to enter _____ in a research study investigating the mechanisms of acidosis and acidosis treatment in calves. This study is part of a larger research program funded by the National Sciences and Engineering Research Council (NSERC). The research findings are to be distributed in scientific journals and are not intended to be used commercially.

Purpose and Objectives of the Study
Acidosis (increased acidity of the blood) is a common complication of diarrheic disease ("scours") affecting calves, and has a significant economic impact on the cattle industry. Acidosis contributes to disease severity, treatment cost and poor outcome of calf scours. Previous research has identified D-lactate produced by intestinal bacteria as an important contributor to calf acidosis. However, the mechanisms leading to accumulation of D-lactate, as well as its metabolism and excretion remain incompletely understood. This study investigates the relative contribution of D-lactate to naturally occurring acidosis in calves, and aims to detect the intestinal "threshold" at which systemic D-lactic acidosis occurs. The ultimate goal of our research program is to design better preventative and treatment strategies in order to reduce the death rate associated with acidosis in calves.

Voluntary Participation
Participation is entirely voluntary, so it is up to you to decide whether or not to enter your calf in this study. Before you decide, it is important for you to understand what the research involves. This consent form will explain why the research is being done, and what will happen to your calf during the study.
You are indicating your consent to participation by signing this form. If you do decide to enter your calf in this study, you are still free to withdraw at any time and without giving any reasons for your decision, however, all data collected about your animal during its enrolment in the study

will be retained for analysis. If you do not wish to participate, you do not have to provide any reason for your decision, nor will you lose the benefit of any veterinary care your calf is receiving.

Confidentiality

While absolute confidentiality cannot be guaranteed, every effort will be made to ensure that the information collected for this study is kept entirely confidential. Your name or any identifying characteristics of your calf will not be attached to any information, nor mentioned in any study report, nor be made available to anyone except the research team. Your identity or that of your calf will not be revealed in any publications arising from this study.

Description of the Research

Basic information including age, breed and gender, presenting complaint and history, diagnosis, outcome (survival or non-survival) and (if applicable) reasons for non-survival is recorded anonymously from the calf's medical record. Blood is collected and is used for standard blood gas analysis and measurement of serum D-lactate concentration. As most of this information is usually obtained as part of the diagnostic work-up, blood volume taken for this study in addition to blood taken for other diagnostic procedures will not exceed 10 mls. A fecal sample will be obtained as soon as possible after admission, and processed for measurement of D-lactate concentration.

Risks and Discomforts

Risks and discomforts are limited to blood collection and fecal sample collection as all other tests are performed on the samples and not the animals themselves. Blood sampling and fecal sampling is a standard diagnostic procedure in veterinary medicine and is considered to inflict minimal discomfort. There is, however, the potential for unforeseeable risks related to sample collection.

Financial implications

There will be no cost to you for entering your calf in this study. You will not be charged for any of the procedures performed solely for research purposes. All costs for the diagnosis, management and treatment of your calf are your responsibility. There will be no reimbursement for entering your animal in this study.

Further Questions, Findings

If you have any questions about this study or desire further information about the study before or after entering your animal, you can contact Dr. Katharina Lohmann at (306) 966 7178.

Signatures

- ☐ I have read or have had this read to me and understood the consent form.
- ☐ I understand that all of the information collected will be kept confidential and that the results will only be used for scientific purposes.
- ☐ I understand that I am entering my animal in this study voluntarily.
- ☐ I freely consent to entering my animal in this study.
- ☐ I have been told that I will receive a dated and signed copy of this form.
- ☐ I am at least 18 years of age and am the legal owner of the animal or am authorized to make decisions regarding this animal on the owner's behalf.

Signature of Owner or Agent Date

Signature of Individual conducting the consent process Date

Witness (optional)

Appendix E

Assessment of Diarrhea Severity

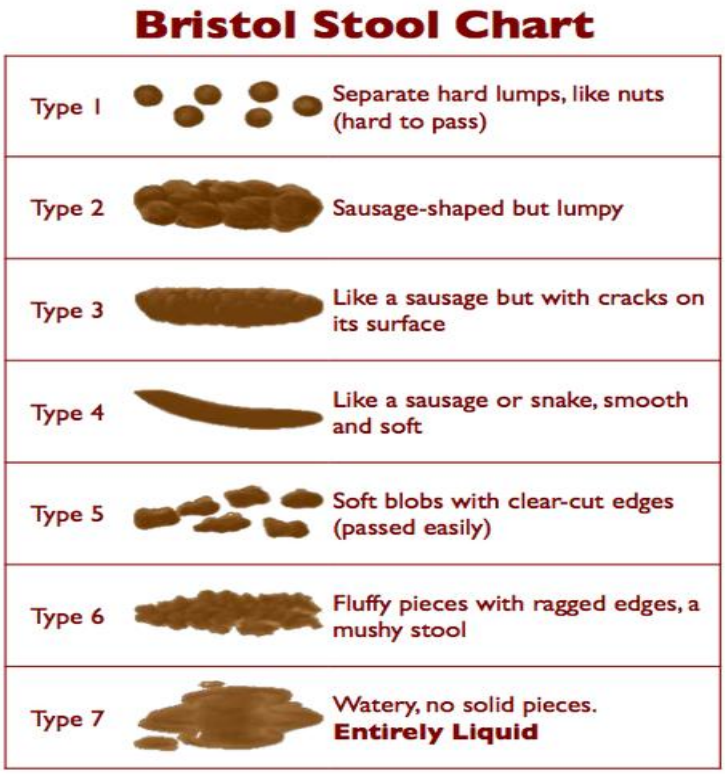
D-Lactic Acidosis in Pediatric Diarrhea

Subject	RUH #	**Stool** Saved? If so, check box & mark type*	**Serum** Saved? If so, check box
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			

Subject	RUH #	**Stool** Saved? If so, check box & mark type*	**Serum** Saved? If so, check box
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			
30			

*Refer to Stool Form Scale below for "Type"

Type 1: Separate hard lumps like nuts
Type 2: Sausage-shape but lumpy
Type 3: Like sausage but with cracks on its surface
Type 4: Like a sausage or snake, smooth and soft
Type 5: Soft blobs with clear-cut edges
Type 6: Fluffy pieces with ragged edges, a mushy stool
Type 7: Watery, no solid pieces



D-Lactic Acidosis in Pediatric Diarrhea

Subject #: _____

1) Stool Consistency Classification System			
Hard and Formed	**Soft but Formed**	**Loose & Unformed**	**Liquid**
			
Having a hard or firm texture and retaining a definite shape like a banana, cigar or marbles	Retains its general shape; has a texture that appears like butter	Lacking any shape of its own; having a texture that appears like hot cereal	Like water

2) Diarrheal Frequency				
	Diarrhea Stools in Preceding 24 hours? Please insert exact #____, and check corresponding box			
Stool Consistency	0 stools	1 – 4 stools	5 – 8 stools	≥9 stools
Loose & Unformed Or Liquid Stool(s)	□	□	□	□

3) Duration of Diarrhea				
	Number of Days with Diarrhea (in previous 13 days)? Please insert exact #____, and check corresponding box			
Stool Consistency	0 days	1 – 2 days	3 - 4 days	≥5 days
Loose & Unformed Or Liquid Stool(s)	□	□	□	□

4) Vomiting Frequency				
	Episodes of Vomiting in Preceding 24 hours? Please insert exact #____, and check corresponding box			
Vomiting	0 episodes	1 episode	2 episodes	≥3 episodes
Yes	□	□	□	□

5) Duration of Vomiting				
	Number of Days with Vomiting (in the previous 13 days)? Please insert exact #____, and check corresponding box			
Vomiting	0 days	1 – 2 days	3 - 4 days	≥5 days
Yes	□	□	□	□

6) Fever				
	Temperature? Please insert exact #____, and check corresponding box			
Axillary	≤37.3°C (≤99.1°F)	37.4 to 38.1°C (99.3 to 100.6°F)	38.2 to 38.9°C (100.7 to 102°F)	≥39.0°C (≥102.2°F)
Yes	□	□	□	□

Appendix F:

Clinical Assessment Scoring and Calf Record Tools

INDIVIDUAL CALF RECORD – THRESHOLD STUDY

Date: **Time:**
Calf #:
Date of birth:
Diarrhea since:
Treatments (if any) including date and time of administration:

Other comments: Blood gas analysis (I-Stat CG8+)

Glucose:		Sodium:	Potassium:	
TCO_2:	iCa:	Hct:	Hb:	
pH at 37 ^oC:		PCO_2:	PO_2:	HCO_3:
BEecf:		sO_2:		

Clinical assessment score:

Physical Appearance and Behavior		**Score for this calf**
0	Normal, bright and alert	
1	Mildly lethargic	
2	Dull, nasal discharge, not interested in food	
3	Recumbent, cannot be roused	
Food and water intake		
0	Normal	
1	Decreased, does not finish meal	
2	Eats less than half of meal	
3	Doesn't eat at all	
Rectal temperature		
0	Normal (T = 38-39)	
1	Fever (39 < T < 40)	
2	Fever (T > 40)	
3	Hypothermia (T < 37.5)	
Character of Feces		
0	Normal	
1	Soft but do not soak through bedding	
2	Watery (soak through bedding)	
3	Watery and animal appears dehydrated (eyes sunken)	
Other		
0	No abnormal findings	
2	Coughing, colic, wobbly etc.	

DESCRIPTION OF THE DAILY CLINICAL SCORING METHOD

Body weight:___ Kg

Depression score (maximum score of 9)

Suck reflex: 0 = strong and coordinated
1 = weak but chewing
2 = absent

Attitude: 0 = Normal behaviour, alert, gets up when approached, interested in surroundings.
1 = Depressed, reluctant to stand, must be stimulated to get up
2 = Unable to stand, even with help. Calf in sternal recumbency
3 = Calf in lateral recumbency (sometimes comatose/collapsed)

Hydration Status Score (maximum score of 2)

0 = Normal hydration, skin tent < 2
1 = Moderate dehydration, eyeball slightly sunken (enophtalmia), and skin tent > 2 s but < 4 s.
2 = Obvious dehydration, eyes sunken with an easily perceptible distance between the eyeball and the eyelid, dry nose, skin tent persists > 5 seconds

Warmth of oral cavity and external limb temperature of the hock and fetlock.

0 = normal (warm)
1 = cool oral cavity and extremity temperature
2 = very cold oral cavity and extremity temperature

Eye skin turgor **Number, size, color and proximity from the limbus of the vessels from the sclera (maximum score of 3)**

0 = Normal (<2), they do not reach the limbus
1= Greater in number (<4), at least 1 reaches the limbus, color is still pink, size is normal
2 = Greater in number (>4), at least 2 reach the limbus, color is red, size is mildly increased.
3 = Greater in number (>6), at least 3 reach the limbus, color is purple, size is greatly increased.

Umbilicus **Pain, size, dryness, presence of pus (maximum score of 3)**

0 = Normal, pencil size, dry, and painless
1 = Bigger than normal, but dry and painless
2 = Bigger than normal, wet or painful
3 = Bigger than normal, with pus draining and evidence of pain (any presence of internal umbilical swelling ranks as a 3 and should be described as involving arteries/urachus or vein)

Fecal Score Consistency (maximum score of 3)

0 = normal (firm, pasty)
1 = Feces softer than normal, semi-solid, but no diarrhea on the tail
2 = liquid diarrhea, but not profuse; wet tail
3 = Profuse watery diarrhea, wet tail, soiled pen, or any indication of blood in feces (but not normal feces with a small amount of blood).

RECORD SHEETS for_____(Diarrhea and Acidosis Trial)

Record Sheet A: Information upon admission

ID Number	Date	Diarrheic or Control?	Date of Birth	Sex	Total Depression Score (Sheet B)	% Dehydration (Sheet C)	Diarrhea Type (Sheet D)	Comments

1. Diet prior to diarrheic episode:
2. Standard of care for _____:

Record Sheet B: Clinical signs upon admission

_____ (insert animal species) Diarrhea and Acidosis Trial

ID Number	Date	Suck*	Menace*	Palpebral*	Tactile*	Ability to Stand*	Total	Comments

*** Please refer to Record Sheet B Scoring System**

Record Sheet B Scoring System

Variable	Method of Assessment	Score	Interpretation
Suck	Index finger in mouth	0	Normal (strong suck, can feel tongue exerting pressure)
		1	Weak suck (can feel little pressure inside mouth, calf not eager to suck)
		2	No suck
Menace	Animal's reaction (blinking) when a researchers hand moves quickly and stops abruptly in front of calf's	0	Normal (blinks strongly and quickly)
		1	Weak (blinking is clearly delayed and calf may not respond to all hand movements)
		2	No menace (animal won't blink in response to hand movement)
Palpebral	Animal's reaction (blinking) when researchers touches the calf's third eyelid Scoring similar to menace	0	Normal (blinks strongly and quickly)
		1	Weak (blinking delayed and calf may not respond to all hand movements)
		2	No menace (animal won't blink in response to hand movement)
Tactile	Pen used to poke the calf on the back	0	Normal (animal twitches strongly and quickly after each poke)
		1	Weak (won't respond to all pokes & twitches are weak)
		2	No tactile response (No detectable response to poke)
Stand	Animal's ability to stand	0	Normal (animal can easily stand on its own)
		1	Depressed ability to stand (animal has to be lifted slightly in order to stand)
		2	Recumbent (Animal cannot stand)

Abeysekara, S. (2009). D-Lactic Acid Metabolism and Control of Acidosis. (PhD thesis, University of Saskatchewan, Saskatoon, Canada). Retrieved from http://library2.usask.ca/theses/available/etd-01202009-120329/restricted/Abeysekara-Saman_PhD_D-lacticAcidosis_ETD.pdf

Kasari, T. & Naylor, J. (1986). Further Studies on the Clinical Features and Clinicopathological Findings of a Syndrome of Metabolic Acidosis with Minimal Dehydration in Neonatal Calves. *Can J Vet Res*, 50, 502.

Sheet C: Dehydration upon admission

To evaluate hydration:

- Skin tenting: pinch a fold of skin (best done on the neck) and count the seconds it takes to flatten. Flattening of skin in less than 2 seconds indicates normal hydration. If skin takes 2 to 6 seconds to flatten, the calf is about 8% dehydrated. Over 6 seconds indicates severe dehydration above 10%.
- Gums: evaluated by looking at their color and feeling them for moisture. Normal gums should be pink and damp but if gums are white and dry this indicates 8 to 10% dehydration.
- Assign % dehydration to each animal ID # upon admission. Record % dehydration in **Record Sheet A**

Dehydration	Symptoms
5-6%	Diarrhea, no clinical signs, strong suckling reflex
6-8%	Mild depression, skin tenting 2-6 seconds, calf still suckling, sunken eyes, weak
8-10%	Calf depressed, laying down, eyes very sunken, dry gums, skin tenting >6 seconds
10-14%	Calf will not stand, cool extremities, skin won't flatten when tented, comatose
Over 14%	Death

Kehoe, S. & Heinrichs, J. (2009). Electrolytes for Dairy Calves. *Cooperative Extension System, The Pennsylvania State University & Department of Dairy and Animal Science*. Retrieved from: http://www.extension.org/pages/Electrolytes_for_Dairy_Calves

Sheet D: Diarrhea Type

Diarrhea

0	Normal (Hard, solid feces)
0.5	Slightly Diarrheic (Feces appears soft)
1	Diarrheic (Feces has no distinct shape, calf appears slightly dehydrated. Electrolytes will be given in its regular feeding.)
1.5	Very Diarrheic (Feces is watery and calf is dehydrated. Blood may be seen in feces. Electrolytes are given to calf.)
2	Extremely Diarrheic Calf (Profuse, watery feces with blood. Calf will be euthanized).